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DIFFERENCES IN SUSCEPTIBILITY TO WHOLE-BODY GAMMA IRRADIATION IN THE LAYERS OF THE RETINA OF BUFO

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Differences in susceptibility to irradiation constitute a problem of biological significance heightened by the fact that they occur in comparable tissues in different groups of animals. We have found that in *Bufo* the cells destroyed under the conditions of our experiments include certain small nerve cells of the brain, of the olfactory membrane, and of the retina. This paper deals with the latter because it offers an especially instructive example of these principles.

Materials and Methods

Recently metamorphosed *Bufo boreas halophilus* with trunk lengths of 10 to 12 mm. were firmly held in the zone of maximum irradiation while being exposed to total-body irradiation by a cobalt^{60} source. The average dosage rate was 950 r/minute. Fixation was by Bouin's fluid. Serial paraffin sections were cut at a thickness of one to five microns. A thickness of three microns was the most suitable. Slides were left over-night in commercial H_2O_2 in order to expose the rods and cones by depigmenting the strands from the pigment layer.

Some material was stained with Delafield's hematoxylin and eosin, but by far more useful was Heidenhain's iron-alum hematoxylin, slides being left one-half hour in each of the two solutions. This stain proved especially valuable not only because of its sharpness but because it stained the pyknotic nuclei most intensely.

Results

Gamma irradiation of 10,000 r, 20,000 r, 30,000 r, and 60,000 r caused destruction to the nerve cells of the inner nuclear and ganglionic layers. The few nuclei surviving after a dose of 60,000 r may be largely identified as those of the fibers of Mueller and the amacrine cells. On the other hand, even 60,000 r within the twenty-four hour survival period of the toads caused no destruction to the outer nuclear layer nor to the rods and cones belonging to them. At the lower irradiation levels the ganglionic layer appeared to be somewhat less susceptible than the inner nuclear layer, but with an irradiation of 60,000 r it, too, was completely destroyed (Fig. 2). The occurrence of structures interpreted as chromosomal vesicles in all

the retinal nuclei is considered to be normal because they are the same in experimental and control animals.

Toads irradiated with 10,000 r or 20,000 r and killed at twenty-four hours (Fig. 4 and Fig. 6) showed considerable destruction of the inner nuclear layer but this was almost complete at 30,000 r and 60,000 r. Toads irradiated at 10,000 r

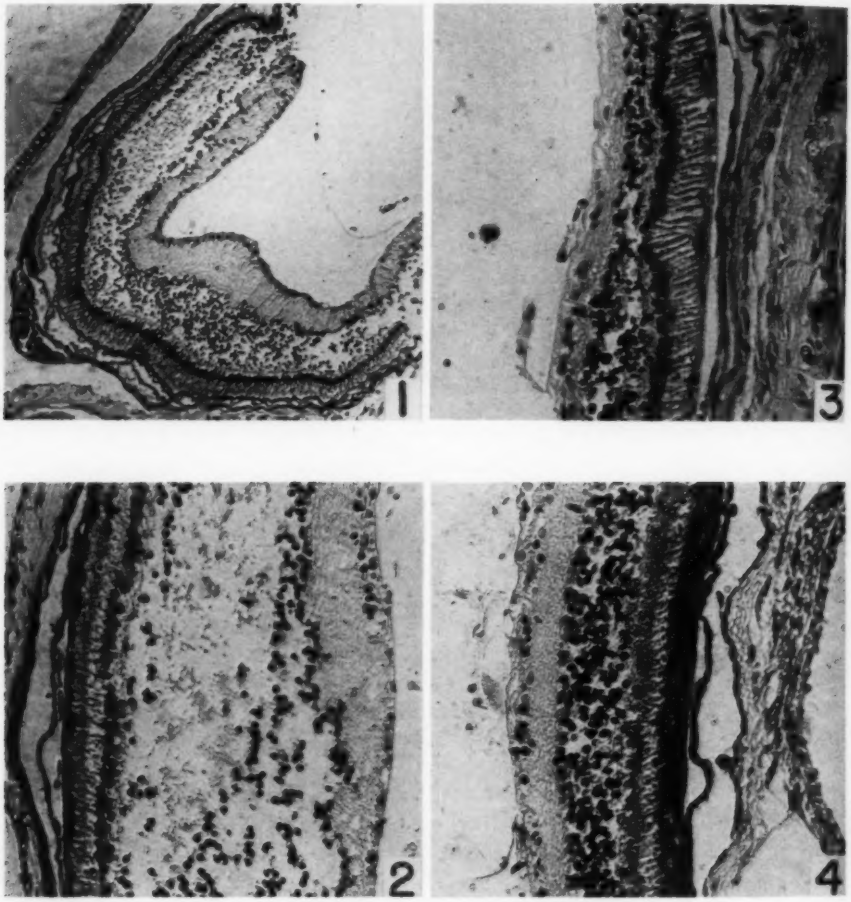

FIGURE 1. The retina at twenty-four hours after being given 60,000 r; × 60.

FIGURE 2. A higher magnification of the area in Figure 1, showing a portion of the more edematous area. × 239.

FIGURE 3. The retina six days after having been given 20,000 r. The thickness of the internal nuclear layer is reduced. The lowest part of the picture is the nearest to the ora serrata. × 329.

FIGURE 4. The retina twenty-four hours after having been given 20,000 r. × 239.

and 20,000 r and killed at an interval of six days after irradiation (Fig. 3 and Fig. 7) showed decided reduction in the thickness of the inner nuclear layer due to resorption of dead nuclei.

With doses of 60,000 r, groups of toads were killed immediately and at short intervals of one hour, two hours, and three hours after irradiation. In general, the effect was a delayed one, pyknosis becoming more and more extensive up to 24 hours. This was observed both after x-ray and gamma irradiation, the results being closely similar. A few cases of pyknosis were seen by the time irradiation was completed. In these early stages pyknosis involved a darkening of the nuclei and only later was there destruction of the cytoplasm. These pyknotic cells occurred throughout the inner nuclear layer of the retina but were most numerous a short distance in from the margin. Not only do the nuclei become deeply pyknotic but decided edema of the retina results. This was localized in the case of lower irradiation dosages, but with 60,000 r the entire retina becomes very heavily pyknotic and edematous (Fig. 1).

Figures 6, 7, 8 and 9 show portions of the retina under different degrees of irradiation. Pyknosis is roughly proportional to the amount of the dose. It is clear that even the highest dose does not affect the outer nuclear layer or the rods and cones, as observed at 24 hours or at six days after irradiation with 20,000 r.

Experiments were performed to show the effect of divided doses on the basis of a 60,000 r total. Irradiation was given on consecutive days and at the following rates: 10,000 r, six times; 20,000 r, three times; and 30,000 r, two times. Destruction of the inner nuclear layer was comparable to that caused by a single dose of 60,000 r. There was a marked reduction in thickness of the inner nuclear layer due to resorption during the course of the experiment (Fig. 8). Divided doses did not cause the amount of edema produced by a single dose.

A very interesting condition is seen at the ora serrata (Fig. 10). The cells of this region show no visible differentiation and there is no sharp line of demarcation between prospective sensory and nerve cell layers, but the nuclei toward the cavity of the eyeball can be followed to the inner nuclear and ganglionic layers, and we consider them to be prospective nerve cell nuclei. It is significant that irradiation renders these highly pyknotic. On the other hand, the nuclei adjacent to the choroid coat, considered to be prospective sensory cells, are not pyknotic. In the region more peripheral to the ora serrata there is likewise no pyknosis in spite of the fact that it is the zone where mitosis had added to the retina, up to a stage shortly before this. It would seem clear that some change has taken place in the prospective nerve cells that renders them especially sensitive to irradiation.

Following the differentiated nerve cell layers central to the ora serrata the distribution of pyknotic nuclei shows that cells of the peripheral portion of the retina are far more readily destroyed than the ones nearer to the center of the retina. At a lower degree of irradiation, 10,000 r and 20,000 r, this is quite evident but at 60,000 r the entire retina is deeply affected.

Discussion

The effects of irradiation upon the retina of amphibians have been chiefly studied in young stages. Brunst (1955) applied x-rays in doses of 1000 r to 8000 r to axolotl larvae from 9 to 65 days of age, the experiment being terminated in 18 to

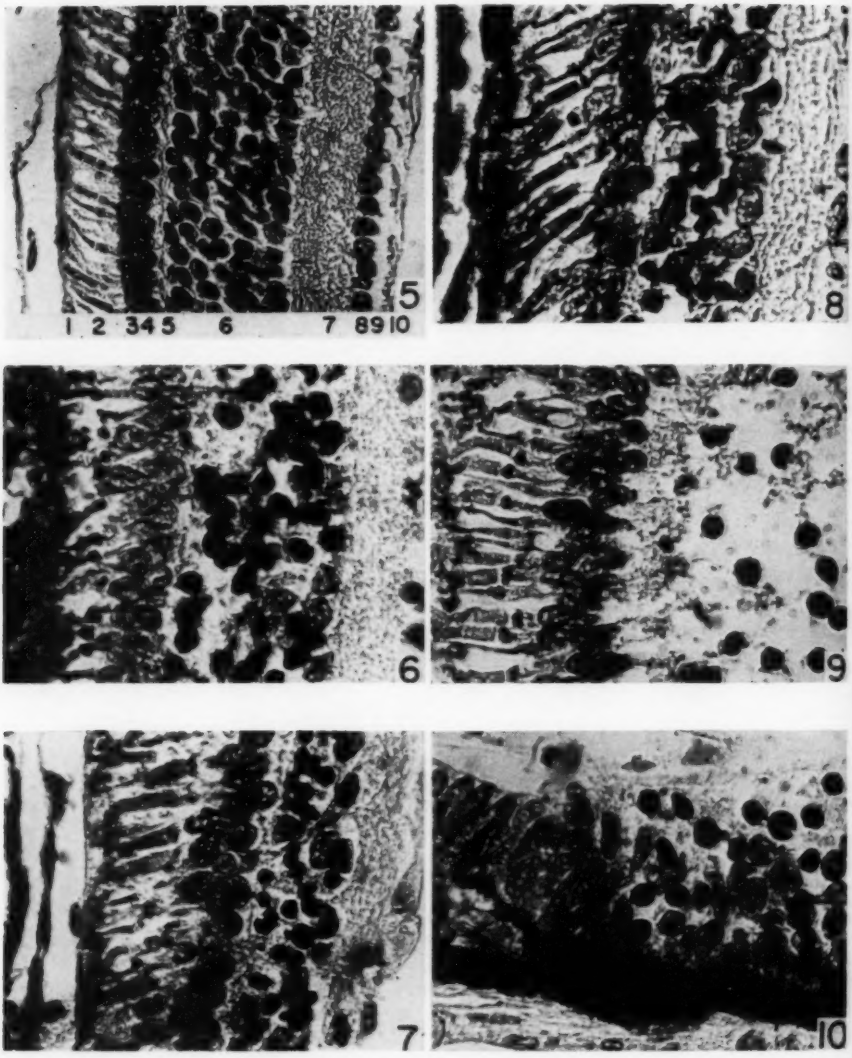

FIGURE 5. Section from the retina of a normal control animal. (1) pigment layer, (2) the rod and cone layer, (3) outer limiting membrance, (4) outer nuclear layer, (5) outer plexiform layer, (6) inner nuclear layer, (7) inner plexiform layer, (8) ganglionic layer, (9) nerve fiber layer, (10) internal limiting membrane. × 1110.

FIGURE 6. The retina twenty-four hours after having been given 20,000 r. × 1402.

FIGURE 7. The retina six days after having been given 20,000 r. Pyknotic nuclei are shrunken and resorbed. The thickness of the inner nuclear layer is reduced as compared to Figure 6. × 1402.

28 days after irradiation. He stated that in all cases the rod and cone layers disappeared first. Eventually the retina degenerated leaving only pigment cells. Brunst (1955) further states (p. 289): "These observations justify the conclusion that the eyes of animals used for this investigation are organs in the process of differentiation and active growth and are therefore, according to the law of Burgonie and Tribondeau, sensitive to roentgen irradiation."

Rugh (1954) finds that in *Ambystoma* larvae 22 mm. in length, x-irradiation of 15,000 r not only causes destruction of mitotic cells, but is equally destructive in regions lying quite apart from them. In a discussion following the reading of this paper, Rugh stated (p. 63), "the rods and cones are separated from the pigment layer but are not individually damaged as are the neuroblast cells. . . . The rods and cones are apparently not relatively sensitive or delicate."

In our own work the rods and cones and the plexiform layers are completely developed (Fig. 5). At the same time this condition has been rather recently attained. The peripheral region of the retina is the youngest part, having been built up as a result of mitotic activity in the region of the ora serrata, as shown by Spear and Glucksman (1941). We have shown that irradiation with 10,000 r and 20,000 r produces very heavy destruction of the inner nuclear and ganglionic layers a short distance central to this region. This leads to the assumption that the susceptibility of these cells is conditioned by their degree of maturity, together with the intensity of the irradiation. In our work we find no evidence that the outer nuclear layer or the rods and cones are affected even by 60,000 r in the 24 hours through which the toads survive. Edema is localized in the case of 10,000 r and 20,000 r irradiation but it is general when 60,000 r is given in a single exposure. It is of secondary importance because it does not appear when 60,000 r is given in divided doses. We have shown that the amount of pyknosis produced by divided doses appears to be roughly equal to that observed when irradiation is given in a single dose. This was shown in *Triturus* by Brunst and Sheremetieva-Brunst (1949).

In sharp contrast to our findings in *Bufo*, the investigators who have irradiated the eyes of mammals have found most extensive injury to the outer nuclear layer, notably to the nuclei of the rods and to the rods themselves. The cones with their nuclei were less affected. This was the finding of Lorenz and Dunn (1950), who exposed newborn mice to 400 r of x-irradiation and killed them at the end of twelve months. Noell *et al.* (1954) gave x-irradiation to the eyes of rabbits, resulting in the destruction of the visual cells while the inner retinal layers were spared. Similar findings were recorded by Brown *et al.* (1955), and Cibis and Brown (1955), who used gamma and x-irradiation upon the monkey, *Macaca rhesus*. With doses of 10,000 r the rods and their nuclei were affected as early as two hours after irradiation. Only when doses exceeded 30,000 r, was there considerable destruction of nerve cells of the inner nuclear layer.

FIGURE 8. This animal was given 10,000 r on 6 consecutive days. The amount of pyknosis is comparable to that produced by 60,000 r in one dose. The thickness of the inner nuclear layer is much reduced by resorption. There is no edema. × 1402.

FIGURE 9. The retina twenty-four hours after having been given 60,000 r. A large amount of edema and many pyknotic nuclei. × 1402.

FIGURE 10. Undifferentiated margin of the retina near the ora serrata, twenty-four hours after 60,000 r was given. The pyknotic nuclei are continuous with the inner nuclear and ganglionic layers. The pigment epithelium is toward the bottom of the picture. × 1402.

Noell (1953, 1955) experimented upon rabbits by injecting sodium iodate, sodium iodacetate, and also by applying oxygen poisoning as well as x-irradiation. All of these were especially injurious to the rods and in a lesser degree to the cones. They all produced quite comparable effects. The nuclei of the outer nuclear layer were largely killed except those belonging to the cones. He found that with the sodium iodate-poisoning, the pigment epithelium was first affected and the injury to the sensory organelles came later, leading to the view that there is a causal relation.

It is clear that in all of the above papers dealing with experiments upon mammals, it was shown that the rods and their cell bodies in the outer nuclear layer were most sensitive to irradiation, while the nerve cells were affected in far less degree. We have shown that just the reverse is true in *Bufo,* within the time limits used. In fact, we find no injury to the rods and cones and their cell bodies in the outer nuclear layer, while on the other hand, the inner nuclear and ganglionic layers show a very high degree of pyknosis. We shall not attempt in this paper to speculate upon the reasons for this difference but the facts are clear enough, and the point is most significant.

Attention is called to the sensitivity of the prospective nerve cells observed at the ora serrata where structural differentiation has not yet taken place. It is evident that in very early stages these prospective nerve cells have already undergone invisible changes that render them vulnerable to irradiation.

Allen (1956) showed that irradiated toads used in this work not only suffered heavy destruction of the nerve cell layers of the retina but also of the nerve cells of the brain.

Summary

1. Gamma irradiation doses of 10,000 r, 20,000 r, 30,000 r and 60,000 r were given to recently metamorphosed *Bufo boreas halophilus.* Toads receiving the two lower dosages were killed at the end of twenty-four hours and at six days. Those receiving the two higher dosages were all killed at twenty-four hours. Cell destruction was found in the inner nuclear and ganglionic layer of the retina but none was found in the outer nuclear layer nor in the rods and cones.

2. Destruction increased as the dosage increased. At doses of 10,000 r and 20,000 r, pyknotic nuclei were most abundant near the marginal portion of the retina, where the cells had been formed more recently than in the central portion. In animals that were given 60,000 r, pyknosis of retinal nerve cells was general and almost complete.

3. Divided daily doses of 10,000 r given six times, 20,000 r three times, and 30,000 r two times produced as much destruction as a single dose of 60,000 r. The amount of edema in these cases was never as great as that which was caused by a single dose of 60,000 r.

4. At the ora serrata where structural differentiation was not yet evident, cells interpreted as prospective nerve cells were destroyed while there was no destruction of prospective sensory cells of the outer nuclear layer.

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LARVAL DEVELOPMENT OF PALAEMONETES PUGIO HOLTHUIS [1, 2]

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Knowledge of the larval development of the marine species of *Palaemonetes* of the eastern United States is limited to Faxon's (1879) account of the development of *P. vulgaris*. This study was based almost wholly on planktonic larvae collected in an area from which two other closely related species, *P. pugio* and *P. intermedius*, have been described since (Holthuis, 1949). Holthuis (1952) feels that even mature adults of these species often have been confused. The larvae, presumably, may be quite similar, although those of the two latter species are unknown.

The problem of distinguishing between decapod species during the larval phase has received relatively little attention. Lebour (1927 through 1943) has found generic, and in some instances specific, taxonomic characters for British decapod larvae, but sometimes only was able to state that larvae of certain species were alike. Gurney (1942) feels that similarity between larvae indicates the relationship between the species. Often larvae of commercially valuable decapods have been studied without consideration of the larvae of related species. Churchill's (1942) account of the zoeae of the blue crab, *Callinectes sapidus*, is thought by Hopkins (1944) to include larvae of another species, possibly *C. ornatus*. Pearson's (1939) description of the development of the white shrimp, *Penaeus setiferus*, has been questioned by Burkenroad (1949) and Heegaard's (1953) descriptions of larvae of the same species are thought by Gunter (editorial comment in Heegaard, 1953) to include larvae of other penaeids.

Among species most studied there has been relatively little agreement on either the form or the number of larval stages. Churchill (1942) found five blue crab zoeae. Hopkins (1943, 1944) found four, but feels that a fifth may exist which has never been found. Sandoz and Rogers (1944) obtained a pre-zoeal blue crab in the laboratory which has not been found in nature. Heegaard (1953) questions the number of white shrimp stages found by Pearson (1939). The number of naupliar stages reported for other species of *Penaeus* varies still more (Hudinaga, 1942; Heldt, 1938). Many of these accounts were based on larvae caught in plankton and have been questioned. The validity of a reconstruction depends upon the ability of the author to recognize species during the larval phase. The difficulty is apparent, especially when another closely related species may exist in the same region.

Intraspecific variation in development among euphausids has been fairly well established (MacDonald, 1927; Fraser, 1936; Boden, 1950, 1951), but variation

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[2] Part of a thesis submitted to the graduate faculty of Duke University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

in decapod larvae has been denied (Gurney, 1942). Heegaard (1953), however, feels that, at least among penaeids, variation in the rate of larval development may result in differences in the number and form of larval intermolts for each species. Some doubt may have been cast on this hypothesis by the paucity and questionable identity of the material on which it was based. The pre-zoeal stage of *Callinectes* reported by Sandoz and Rogers (1944) has usually been dismissed as abnormal.

The present paper is first a description of the larval development of *Palaemonetes pugio* Holthuis based on observations of larvae reared in the laboratory. The larvae and the development are compared to that of *Palaemonetes vulgaris* (Say) reared concurrently. Finally, the considerable variation in the number and structure of larval intermolts, found in the development of both species, is described.

The author wishes to express his sincere appreciation to Professor C. G. Bookhout, under whose guidance and direction this work was done and who read and suggested improvements in this manuscript. Thanks are also due to Dr. T. R. Rice, who supplied stocks of all unicellular algae used, and to Dr. Fenner A. Chace, Jr., who provided working space and specimens of *Palaemonetes* from the collection of the U. S. National Museum.

Methods

Mature adult *P. pugio* and *P. vulgaris* are abundant in the vicinity of the Duke University Marine Laboratory at Beaufort, N. C., from late April until mid-October. Egg-bearing females were caught in dip nets and held individually in dishes of sea water in the laboratory until the eggs hatched, after which they were preserved for later identification and reference.

Groups of 10 newly-hatched larvae, all from the same clutch of eggs, were placed in clean four-inch finger bowls of sea water. The water was changed only if evidence of cloudiness appeared. The bowls of larvae were placed near north windows but not in direct sunlight. Because of other experiments which required constant illumination in an adjacent part of the laboratory, the room was never completely dark. No method of controlling the temperature of the building was available during the summer months. Most of the larvae were reared at temperatures which ranged from about 25 to 27° C. and none at lower than 20° C.

The diet of each larva was the same during its lifetime, but a variety of different foods or combinations of foods was offered to various larvae. Some were not fed. Other individuals received daily rations of either a species of unicellular algae, or a combination of species. Algae used included two species of *Nitzschia* and one species of each of the following genera: *Chlamydomonas, Thorocomonas, Nannochloris, Porphyridium* and *Pyramimonas.* Some larvae were fed species of algae combined with zooplankton which had been obtained by net and first killed by immersion in distilled water to prevent the fortuitous inclusion of living larvae similar to those being studied. The diet of some other larvae consisted of freshly killed zooplankton alone. A few larvae were fed on chaetognaths removed from the plankton and some others were fed tiny bits of the visceral mass of the mud snail, *Nassarius obsoletus.* The remaining larvae were fed living *Artemia* nauplii. Food was added to the bowls and uneaten food was removed daily.

Each larva was inspected daily under the low power of a stereoscopic binocular microscope. A record of molting by each larva was kept. Only the presence of

all or a large part of an exuvium or cast exoskeleton was accepted as evidence that a larva had molted. Morphological changes in larvae established which individuals had undergone the molt.

Camera lucida drawings were made of entire living larvae which had been anesthetized with ethyl carbamate and placed on slides beneath supported cover slips. These preparations were then sealed with oil. Larvae lived for as long as two hours under observation. Individual appendages from alcoholic specimens were studied and drawn.

Finally, adult females from which larvae were obtained were identified after comparison with type and other material at the U. S. National Museum.

Results

The number of individuals of each species which survived each of the several molts is given in Table I. Only those larvae which were fed diets containing some animal tissue were able to survive. The number of ecdyses and, conversely, the number of intermolts or so-called larval stages were not constant among individuals of the same species.

In general, the differences were slight and the similarities great between the larvae of *P. pugio* and those of *P. vulgaris*. In some instances, preserved larvae were identical. Among living larvae, however, a difference in the distribution of chromatophores on the ventral surface of the abdomen proved to be an invariable index to species. Larvae of *P. pugio* bear, on the sternites of abdominal somites

Table I

Number of larvae of Palaemonetes pugio and Palaemonetes vulgaris which survived each molt in the laboratory and the number of postlarvae obtained by metamorphosis. Column headings indicate diet of larvae

Molt number	*Palaemonetes pugio*					*Palaemonetes vulgaris*			
	No food	Unicellular algae	Algae plus animal tissue	Animal tissue	*Artemia* nauplii	No food	Unicellular algae	Algae plus animal tissue	*Artemia* nauplii
0	60	280	608	732	100	80	100	667	390
1	42	162	491	464	92	0	1	30	324
2	0	0	143	177	87		0	15	282
3			77	84	87			13	261
4			54	70	85			11	227
5			47	50	82			11	191
6			37	36	82			9	149
7			29	28	69			8	131
8			24	25				8	
9			18	14				8	
10			17						
11			17						
Number postlarvae	0	0	16	6	65	0	0	6	122

2 and 3, pairs of chromatophores. The larvae of *P. vulgaris* bear chromatophores on the sternite of abdominal somite 3, but lack pigment spots on abdominal sternite 2.

Although the individual larvae and the general pattern of development of *P. pugio* are nearly identical to that of *P. vulgaris*, differences between individuals of the same age and molting history and differences in the duration and tempo of larval development were observed in each species. Larvae which passed through the greatest number of molts during development showed the least morphological change after each molt. The structural characteristics of the individual larva were more readily associated with its total length than with its age or the number of molts completed. Although larvae which were of the same age often differed from one another in extent of development completed, those of about the same size were quite similar in structure regardless of the age or previous molting history. All larvae were alike upon hatching. This similarity began to disappear after the second molt. Considerable variation was found after the third molt, but, at the end of larval development, all larvae again resembled one another. The sequence or order of developmental events was the same for all larvae. The number of steps or stages passed through varied.

Differences in the form of larvae were accompanied by variation in the actual rate of development. Larvae fed living *Artemia* nauplii usually metamorphosed at the seventh molt which occurred about two weeks after hatching. Others, however, required from two to four weeks and as many as 13 molts to complete larval development. The variation observed, therefore, was primarily one of tempo.

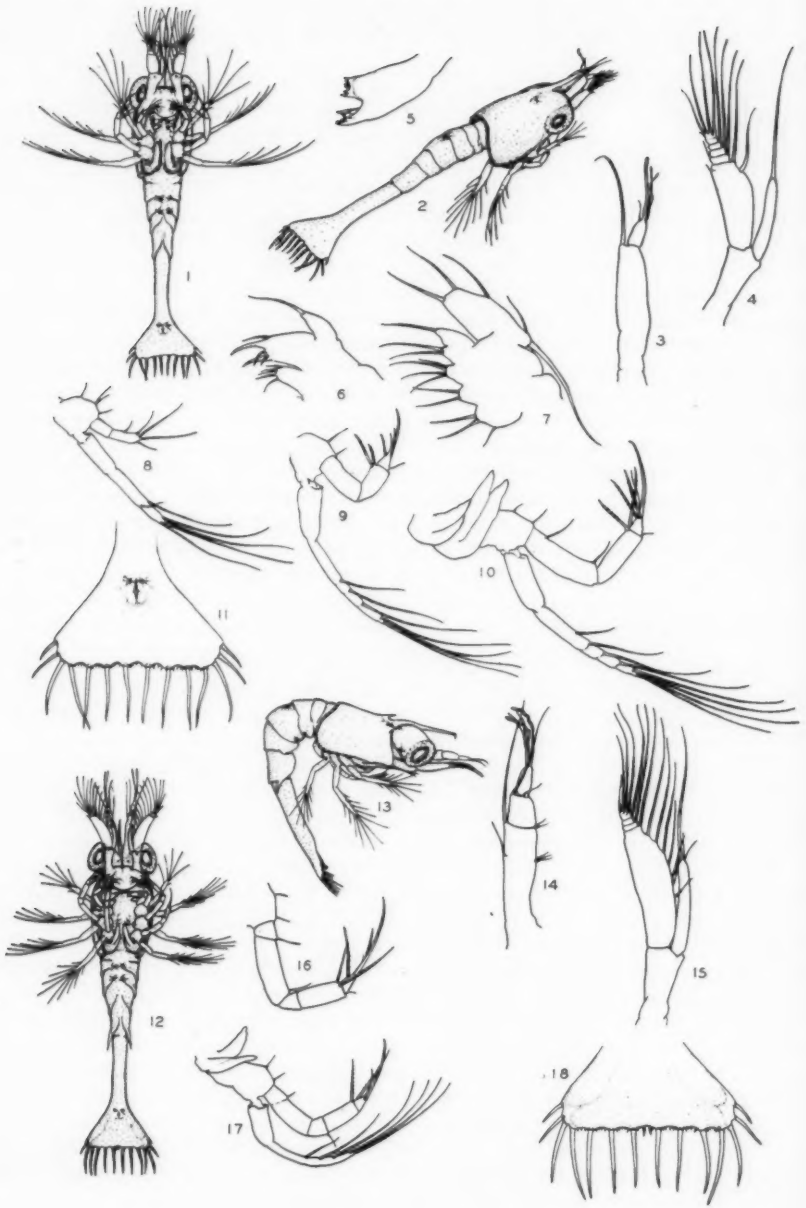
Larvae of Palaemonetes pugio

The eggs carried by adult females were from 0.5×0.6 to 0.6×0.9 mm. in size. They usually hatched within a few days, but always within two weeks or not at all. The prezoeal molt occurs immediately before hatching (Burkenroad, 1947), and the larva emerges from the egg as a zoea.

First zoea (Figs. 1–11): total length about 2.6 mm. Carapace, rostrum and abdomen without spines or teeth. Rostrum curved slightly downward at end. Abdomen of 6 somites, the last of which is not separate from the fan-shaped telson (Fig. 11). Telson with 14 spines. Eyes sessile, contained beneath carapace.

Antennule (Fig. 3) simple; the single basal segment bears terminally a long seta and a short outer flagellum; outer flagellum with three slender and one stout aesthetes and a short seta. Antenna (Fig. 4) biramous; basis unsegmented; flagellum of one segment, shorter than scale with a long terminal seta; scale of a long basal segment, which is convex on inner side, and four short terminal segments, with nine long setae on inner side and three short setae outside near tip.

Mandible (Fig. 5) without palp; incisor process with three teeth at tip; molar process with fine-toothed cutting edge; two movable teeth in angle between molar and incisor processes. First maxilla (Fig. 6) uniramous; coxa with five inwardly directed spines; basis with three spines and two teeth; endopod simple, palp-like, with a terminal seta. Second maxilla plate-like, biramous; protopod three-lobed, armed with three, two and four setae; endopod unsegmented, bears on a lobe near the proximal end two and terminally one setae; exopod a flattened gill bailer with three setae anteriorly, one laterally and one posteriorly.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First maxilliped (Fig. 8) biramous; coxa reduced; basis with four medially directed setae; endopod two-segmented, the distal segment with four terminal and one median setae; exopod longer than endopod with four apical and two sub-apical setae. Second maxilliped (Fig. 9) biramous; coxa reduced; basis with two setae; endopod three-segmented, with two strong spines at junction of ultimate and penultimate segments, ultimate segment with two smaller spines, a seta and a strong terminal claw; exopod longer than endopod with a cylindrical proximal and a flattened paddle-like distal segment which bears four apical and three pairs of sub-apical setae. Third maxilliped (Fig. 10) biramous, larger than second maxilliped, but generally similar to it; endopod with two setae on proximal segment; exopod with four apical and three or four pairs of sub-apical setae.

First and second pereiopods (Fig. 10) rudimentary. Other appendages lacking.

Prominent groups of red and yellow-green chromatophores located dorsally at bases of eyes and at junction of abdominal somites 2 and 3. Paired groups of chromatophores ventrally on basal segment of antenna, on labrum, on thoracic sternites 1 and 8, on abdominal sternites 2 and 3 and on telson just anterior to anus.

This larva corresponds very closely to the first zoea of *P. vulgaris* and to Faxon's (1879) description of the first stage larva of that species. It differs from the larva of *P. vulgaris* chiefly in the presence of a pair of chromatophores on abdominal sternite 2, which are lacking from the latter species. The basal segment of the antennal scale of *P. vulgaris* is less convex on its inner side and the scale is narrower than in *P. pugio*.

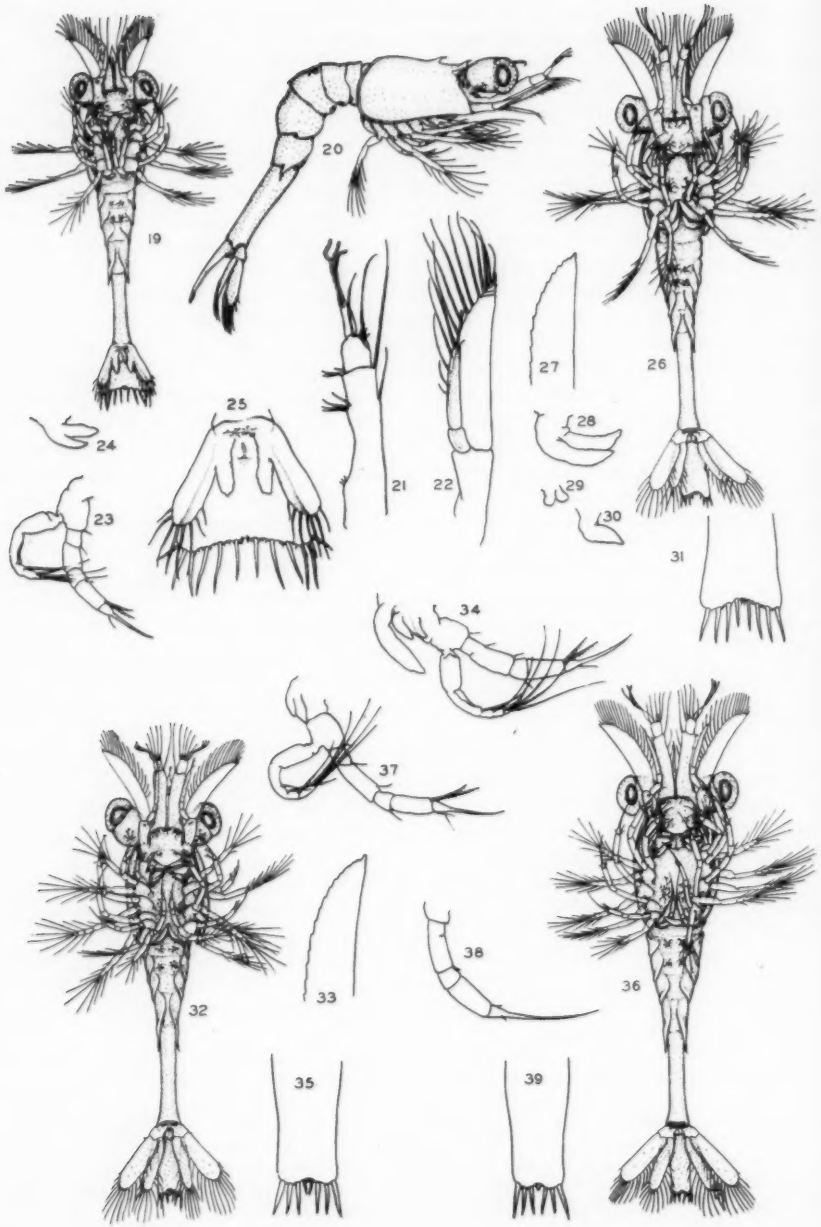
Second zoea (Figs. 12–18): length about 2.8 mm. Differs from first zoea in the following: Carapace with supra-orbital and branchiostegal spines. Rostrum recurved at tip and with one dorsal rostral tooth located on the carapace just behind orbit. Pleurum of fifth abdominal somite terminates as a posteriorly directed tooth. Telson (Fig. 18) with 14 large and two minute spines. The outlines of the uropods often visible within telson. Eyes stalked with chromatophores on postero-ventral side of stalk.

Antennule (Fig. 14) with peduncle segmented, segments marked by long setae on inner side, a long and a short seta on distal end of peduncle; outer flagellum with two slender and two stout aesthetes. Antennal scale (Fig. 15) with three short segments at the distal end and 14 setae; antennal flagellum terminates in a long and two short setae.

First maxilla with four teeth and three spines on basis. Exopod of second maxilla with five anterior setae. Endopod of third maxilliped (Fig. 16) five-segmented.

First pereiopod (Fig. 17) biramous; coxa reduced; basis with two setae; endopod five-segmented, ischium, carpus and dactylus with a seta each, two stout spines at junction of propodus and dactylus, dactylus terminates in a strong claw; exopod as in third maxilliped.

PLATE I. Larval development of *Palaemonetes pugio*. Entire larvae ×19; appendages ×54. Figures 1–11 of first zoea. Fig. 1: ventral view. Fig. 2: dorsolateral view. Fig. 3: antennule. Fig. 4: antenna. Fig. 5: mandible. Fig. 6: first maxilla. Fig. 7: second maxilla. Fig. 8: first maxilliped. Fig. 9: second maxilliped. Fig. 10: third maxilliped and first and second pereiopods. Fig. 11: telson. Figures 12–18 of second zoea. Fig. 12: ventral view. Fig. 13: lateral view. Fig. 14: antennule. Fig. 15: antenna. Fig. 16: endopod of third maxilliped. Fig. 17: first and second pereiopods. Fig. 18: telson.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The second zoea corresponds to the second zoea of *P. vulgaris* and to Faxon's description of the second stage larva of that species. The second zoea of *P. vulgaris* differs from the corresponding larva of *P. pugio* in lacking chromatophores on abdominal sternite 2. All larvae which had molted once were in the form described.

Third zoea (Figs. 19–25): total length about 3.2 mm. Differs from the previous larva in the following: Sixth abdominal somite separate from telson. Telson (Fig. 25) narrower than before, armed with 12 large and two small spines.

Antennular peduncle (Fig. 21) with two long setae ventro-distally, two long setae on inner side, and, on a protuberance near the proximal end which will be the stylocerite, three short setae; a rounded prominence bearing three or four short setae and located dorsally near the distal end of the peduncle is the antennular lobe; outer flagellum with three stout aesthetes. Antennal scale (Fig. 22) with two short segments at distal end and 15 setae; antennal endopod separated into a short peduncle and an unsegmented flagellum which terminates in two short and two minute setae.

Second pereiopod (Fig. 23) biramous; coxa reduced; basis with at least one seta; endopod five-segmented, first and last segments with setae, two stout spines arise from junction of propodus and dactylus, dactylus terminates in a strong claw; exopod shorter than endopod, similar in structure to other thoracic exopods. Third pereiopod (Fig. 24) biramous, rudimentary.

Uropod (Fig. 25) biramous, unsegmented, with rudimentary inner ramus; outer ramus with 7 or 8 setae.

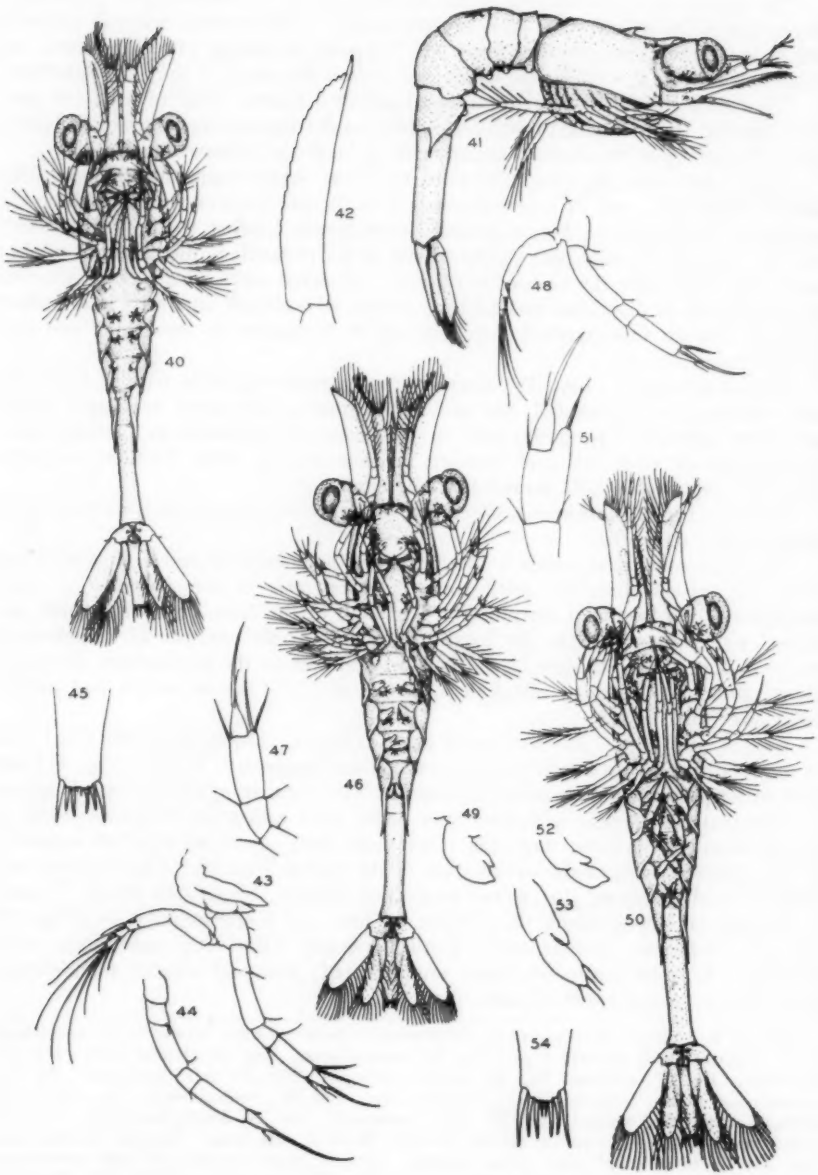
The third zoea corresponds to the third zoea of *P. vulgaris* and to Faxon's third stage. It differs from the third zoea of *P. vulgaris* in the presence of chromatophores on abdominal sternite 2. Variation in the form of larvae which had molted twice was noted in the total length and in the number of rudimentary pereiopods present. Larger larvae had, in addition to the appendages described above, rudiments of third and fourth pereiopods. All larvae which had molted twice were third zoeae.

Fourth zoea (Figs. 26–31): length about 3.5 mm. Differs from the third zoea in the following: Two dorsal rostral teeth on the carapace. Telson (Fig. 31) but little wider posteriorly than anteriorly, armed with eight stout and two small spines.

Antennular peduncle with four long distal setae and a protuberance which is the rudiment of the inner flagellum. Antennal basis separated into two segments by an oblique fissure at the articulation of the peduncle; scale (Fig. 27) not segmented, its disto-lateral tip (spine) projecting slightly, blade with 16 or 17 setae.

Second pereiopod larger than before. Third and fourth pereiopods (Figs. 28 and 29) biramous, rudimentary. Fifth pereiopod (Fig. 30) uniramous, rudimentary. Uropod biramous; basis unsegmented; endopod shorter than exopod bears 8 setae; exopod with 12 setae.

PLATE II. Larval development of *Palaemonetes pugio*. Entire larvae × 19; appendages × 54. Figures 19–25 of third zoea. Fig. 19: ventral view. Fig. 20: lateral view. Fig. 21: antennule. Fig. 22: antenna. Fig. 23: second pereiopod. Fig. 24: third pereiopod. Fig. 25: uropods and telson. Figures 26–31 of fourth zoea. Fig. 26: ventral view. Fig. 27: tip of antennal scale, setae omitted. Fig. 28: third pereiopod. Fig. 29: fourth pereiopod. Fig. 30: fifth pereiopod. Fig. 31: end of telson. Figures 32–35 of fifth zoea. Fig. 32: ventral view. Fig. 33: tip of antennal scale, setae omitted. Fig. 34: third, fourth and fifth pereiopods. Fig. 35: telson. Figures 36–39 of sixth zoea. Fig. 36: ventral view. Fig. 37: third and fourth pereiopods. Fig. 38: fifth pereiopod. Fig. 39: telson.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The fourth zoea of *P. pugio* corresponds to the fourth zoea of *P. vulgaris* but has no equivalent in Faxon's descriptions of the larvae of that species. The fourth zoea of *P. pugio* differs from that of *P. vulgaris* in the distribution of abdominal chromatophores and in the projecting tip of the spine of the antennal scale, which, in *P. vulgaris* larvae, does not extend beyond and free of the blade. Not all larvae which had molted three times correspond to the fourth zoea as described here. Among those larvae which molted the least number of times during development, this form was not represented by any intermolt.

Fifth zoea (Figs. 32–35): total length about 3.5 mm. The fifth zoea differs from the fourth in the following: Telson (Fig. 35) narrower posteriorly than anteriorly, armed with six stout and two slender spines. Antennular peduncle with 5 long setae distally; inner flagellum a separate segment tipped with a short seta. Antennal scale (Fig. 33) with 19 setae, tip projecting; flagellum divided into a short proximal and a longer distal segment.

Mandible with three serrate movable teeth. Basis of first maxilla with five teeth and three setae. Middle lobe of basis of second maxilla with three setae; exopod with six to seven anterior setae.

Third pereiopod (Fig. 34) biramous; coxa reduced; basis with at least one seta; endopod five-segmented, ischium, merus, carpus, and dactylus with setae, two stout spines at junction of propodus and dactylus, dactylus terminates in a claw; exopod shorter than endopod with four apical and two pairs of sub-apical setae. Fifth pereiopod longer than fourth (Fig. 34), both rudiments. Uropodal endopod nearly as long as exopod, with 13 setae; exopod with a short seta on outside near proximal end and a short tooth in dorso-lateral corner, with 16 long setae.

The fifth zoea of *P. pugio* differs from the fifth zoea of *P. vulgaris* as do fourth zoeae. There is no corresponding larval stage in Faxon's account of development of *P. vulgaris*, but a larva of this form was obtained by molt and considered abnormal by Faxon. This form may be skipped in the development of either species.

Sixth zoea (Figs. 36–39): total length about 3.7 mm. Differs from the previous larva in the following: Antennular peduncle with three plumose setae on inner side, angle of stylocerite is acute. Antennal scale with 20 plumose setae. Fifth pereiopod (Fig. 38) uniramous; coxa reduced; short setae on merus, propodus and dactylus, dactylus terminates in a long claw. Uropodal exopod with 16, endopod with 13 or 14 setae.

The form described as the sixth zoea may be present in the development of either *P. pugio* or *P. vulgaris*. The species differ in the ways previously discussed. This form is not represented among Faxon's larval stages, but was seen by him and considered abnormal. This form may be skipped in the development of either *P. pugio* or *P. vulgaris*.

Seventh zoea (Figs. 40–45): total length about 4.4 mm. The seventh zoea

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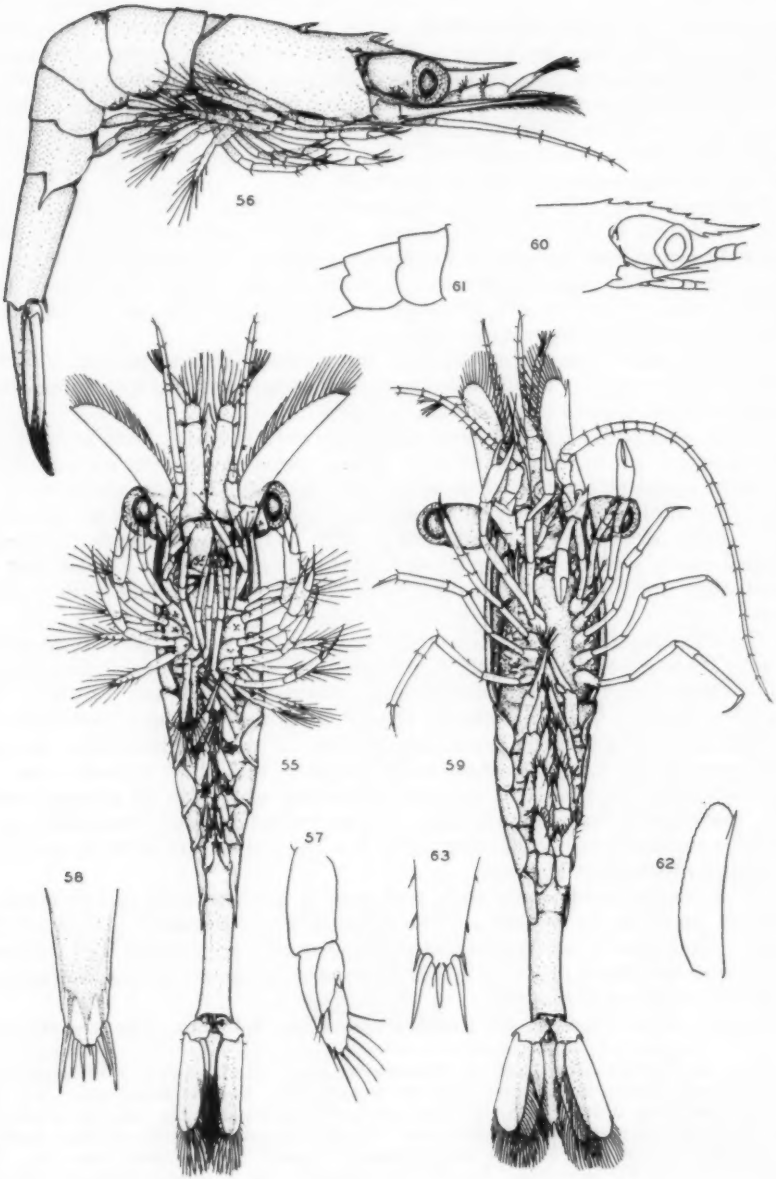
PLATE III. Larval development of *Palaemonetes pugio*. Entire larvae × 19; appendages × 54. Figures 40–45 of seventh zoea. Fig. 40: ventral view. Fig. 41: lateral view. Fig. 42: antennal scale, setae omitted. Fig. 43: third and fourth pereiopods. Fig. 44: fifth pereiopod. Fig. 45: telson. Figures 46–49 of eighth zoea. Fig. 46: ventral view. Fig. 47: first cheliped. Fig. 48: fourth pereiopod. Fig. 49: second pleopod. Figures 50–54 of ninth zoea. Fig. 50: ventral view. Fig. 51: first chela. Fig. 52: first pleopod. Fig. 53: second pleopod. Fig. 54: telson.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differs from the sixth in the following: A minute anal spine present. Antennular peduncle with five setae on inner side; outer antennular flagellum with three stout and one slender aesthetes. Antennal scale (Fig. 42) with 20 setae. Fifth pereiopod with a seta on each segment.

First to fifth pleopods represented by small uniramous buds.

The seventh zoea of *P. pugio* differs from the seventh zoea of *P. vulgaris* as the sixth zoeae differ and in regard to a small tooth on the ventral side of the antennular peduncle of the latter species. This tooth appears later in the development of *P. pugio* and is smaller than in *P. vulgaris*. Considerable variation in the development of the last three pereiopods was found in larvae first having pleopod buds. Among those larvae which, in their development, had skipped the forms described as fourth, fifth and sixth zoeae, pereiopods 3, 4 and 5 were rudiments. Among other larvae pereiopod 5 was still rudimentary at the time of the appearance of pleopod buds. The seventh zoea described here corresponds most closely to Faxon's fourth larval stage of *P. vulgaris*. Larvae of either species may have molted three, four, five or six times when the form described as the seventh zoea is achieved.

Eighth zoea (Figs. 46–49): total length about 4.9 mm. The eighth zoea differs from the previous larva in the following: The anal spine is stronger. The antennular peduncle bears, about mid-way of the proximal segment on the ventral side, a small tooth, 7 setae on inner side, 6 setae at distal end. Antennal basis with a tooth on ventral side at junction of scale; scale with 23 setae; flagellum of three or four segments.

Propodi of first and second pereiopods (Fig. 47) swollen and protuberant at inner distal corner, forming, with dactylus, the beginning of a chela. Fourth pereiopod (Fig. 48) biramous; coxa reduced, basis with a seta; endopod five-segmented, ischium, merus and dactylus with one seta each, carpus with two setae, junction of propodus and dactylus with three spines, dactylus tipped with a claw. Fifth pereiopod with a short spine arising from the middle of dactylus.

First to fifth pleopods (Fig. 49) small, biramous, rudimentary. Uropodal exopod with 20, endopod with 18 setae.

The eighth zoea of *P. pugio* corresponds to the eighth zoea of *P. vulgaris* from which it differs in the ways previously stated. This form corresponds to Faxon's fifth larval stage of *P. vulgaris*. There was considerable variation in the development of the pleopods, but all had non-setose, biramous pleopod rudiments.

Ninth zoea (Figs. 50–54): length about 5.1 mm. Differs from the previous larva in the following: Rostrum setose in the angle of the anterior tooth. Outer antennular flagellum with four equal, sub-apical aesthetes and a slender aesthete arising from mid-way of the segment. Antennal flagellum longer than scale, five-segmented; scale with 26 plumose setae. Entire outer edge of exopod of second maxilla setose. First maxilliped with a simple epipod, basal portion enlarged with 8 setae, proximal segment of exopod with two setae.

PLATE IV. Larval development of *Palaemonetes pugio*. Entire larvae and postlarva × 19; larval appendages × 54. Figures 55–58 of tenth (last) zoea. Fig. 55: ventral view. Fig. 56: lateral view. Fig. 57: second pleopod. Fig. 58: telson. Figures 59–63 of postlarva. Fig. 59: ventral view. Fig. 60: lateral view of rostrum and anterior portion of cephalothorax, × 19. Fig. 61: lateral view of fourth and fifth abdominal pleurae, × 19. Fig. 62: antennal scale, setae omitted, × 19. Fig. 63: telson, × 54.

First and second pereiopods chelate (Fig. 51), the fixed finger tipped by the two spines formerly located at junction of propodus and dactylus. First to fifth pereiopods with arthrobranchs.

First to fifth pleopods biramous. First pleopod (Fig. 52) rudimentary. Second (Fig. 53) to fifth pleopods with sparsely setose exopods. Uropodal exopod with 24, endopod with 17 setae.

The ninth zoea of *P. pugio* differs from the ninth zoea of *P. vulgaris* as previously described. The ninth zoea corresponds to Faxon's stage 6, first intermolt. Variation was noted in the extent of development of the inner ramus of the pleopods, the recurvature of the rostral tip, and the extent of development of the chelae.

Tenth zoea (Figs. 55–58): total length about 6.3 mm. The tenth zoea is the form representing full larval development. It differs from the previous larva in the following: Tip of rostrum curved slightly upward. Telson (Fig. 58) slender posteriorly, armed as before. Inner antennular flagellum with six aesthetes and two short apical setae. Antennal flagellum of 7 segments; scale with 30 setae, its tip projecting or not. Mandible with four or five movable teeth in angle between incisor and molar processes. Basis of first maxilla with 6 teeth. Exopod of second maxilla fringed with 29 setae.

Basis of first maxilliped with 9 setae and a bilobed epipod; proximal segment of exopod with 5 or 7 setae. Pereiopods essentially as before.

First pleopod with rudimentary endopod. Second (Fig. 57) to fifth pleopods biramous with setose exopods and endopods; endopods with small *appendices internae*. Uropodal exopod with 29 and endopod with 26 plumose setae.

The tenth zoea of *P. pugio* differs from the tenth zoea of *P. vulgaris* chiefly in the chromatophores of the ventral abdomen as previously discussed. This form corresponds to Faxon's sixth larval stage, intermolts 2 and 3. Some variation in the extent of development of the *appendices internae* of the pleopod endopods was noted.

Postlarva (Figs. 59–63): length about 6.2 mm. Rostrum shorter than antennal scale, with six dorsal teeth, the first of which is on the carapace directly over the posterior margin of the orbit, and two ventral teeth, the first of which is directly beneath the last dorsal tooth; tip of rostrum free of teeth (Fig. 60). Carapace with antennal and branchiostegal spines. Posterior margins of abdominal pleurae rounded (Fig. 61). Anal spine present. Telson (Fig. 63) with a tooth extending back from the mid-point posteriorly, two pairs of terminal spines of which the inner pair are longer, a pair of setae mid-ventrally near the distal end, and two pairs of dorso-lateral spines about ½ and ¾ of the way from the proximal end.

Antennular peduncle of three segments; stylocerite less than ½ the length of the basal segment of peduncle; antero-lateral spine of basal segment exceeding anterior margin of segment; inner side of peduncle with 10 setae; basal segment containing a statocyst and a short ventral tooth. Inner antennular flagellum simple, five-segmented. Outer antennular flagellum four-segmented, bearing on the anti-penultimate segment two, and on the penultimate segment three aesthetes, and a tuft of setae on the ultimate segment. Length of antennal scale (Fig. 62) about four times its width, outer margin slightly concave; anterior end of spine projects free of blade and is slightly shorter. Antennal flagellum over half of total length.

Mandible strong, incisor process stouter than in larval mandible; teeth of molar process large, forming a triangular surface, with or without movable teeth in angle.

Basal portion of first maxilla bilobed, each lobe bearing on its inner surface numerous coarse setae; endopod palp-like. Basal portion of second maxilla bilobed, each lobe bearing on its inner surface numerous coarse setae; endopod unsegmented with neither lobes nor setae; exopod setose around edge.

Basal portion of first maxilliped large, bilobed, the lobes with coarse setae directed inwardly; endopod reduced, bears two apical setae; exopod with six setae on proximal segment and four long setae at tip of distal segment; epipod large, bilobed. Second maxilliped with five-segmented endopod, ultimate and penultimate segments wider than long, armed with coarse spines; exopod with two setae; epipod small, bilobed. Third maxilliped with four-segmented endopod, coarsely setose throughout; endopod reduced; epipod tiny, bilobed.

First pereiopod chelate, somewhat stouter and shorter than second pereiopod; exopod a rudiment or lacking. Second pereiopod chelate, cutting edges of chela without serrations or teeth. Carpus shorter than palm; exopod rudimentary if present. Third, fourth and fifth pereiopods not chelate, exopods rudimentary if present. Arthrobranchs at bases of pereiopods.

Endopod of first pleopod rudimentary. Endopods of pleopods 2 to 5 with *appendices internae*. Uropodal exopod sparsely setose along outer edge with a tooth and a movable spine in the disto-lateral corner, numerous setae around the tip and on inner edge; endopod with setae on inner edge and around tip.

The distinctive distribution of chromatophores which characterized the larva is lost in the postlarva. The young prawn appears colorless to the unaided eye, but actually has numerous tiny chromatophores on the cephalothorax and abdomen. The postlarva of *P. pugio* is strikingly similar to the postlarva of *P. vulgaris*. The characters used to separate adults of these species are undeveloped in postlarvae. No attempt is made here to offer characters by which the two species may be distinguished at this stage of development. The postlarva corresponds to Faxon's seventh stage.

The sequence of larval intermolts

The descriptions of zoea larvae given above were based on the structure of individuals reared in the laboratory. Since the number of molts during development is not constant, it is obvious that certain of the described forms may be omitted in the life history of any individual. The relationship between the number of molts and the structure of intermolts observed in the laboratory is given in Table II. Column 4 of Table II shows that, for some larvae, the sequence of successive intermolts was that given in the text descriptions. Columns 5 to 9 show progressive omission of more of the described forms. The data suggest a relationship between diet and the number and form of larval intermolts.

Discussion

Larvae of *Palaemonetes* reared in the laboratory were structurally identical to those from nature described by Faxon (1879). This resemblance extended even to certain larvae considered abnormal by Faxon but shown by rearing to belong to series of intermolts which ultimately metamorphosed to produce normal postlarvae. The distinctive distribution of abdominal chromatophores, which was found to be diagnostic for the species treated in this paper, was not noted by Faxon

TABLE II

The relationship between molting and form of Palaemonetes pugio and Palaemonetes vulgaris larvae fed various diets. Numbers in column 1 refer to text descriptions. Numbers in parentheses refer to described variations. The sequence of forms through which larvae pass in development is given in columns 4 to 9.

Zoea larva No.	Approx. total length (mm.)	Recognition character	Non-living animal plus unicellular algae		Intermolt number Non-living animal		*Artemia* nauplii	
1	2.6	Sessile eyes	1	1	1	1	1	1
2	3.0	Stalked eyes	2	2	2	2	2	2
3	3.2	Telson and uropods	3	3	3			
(3)	3.3	4th and 5th pereiopod rudiments				3	3	3
4	3.5	2 dorsal rostral teeth	4	4	4	4		
5	3.5	3rd pereiopod	5	5				
6	3.7	5th pereiopod	6	6	5			
7	4.4	Pleopod buds	7	7	6	5	4	4
8	4.9	Pleopod rudiments	8		7	6		
(9)	5.0	Chelae		8			5	5
9	5.1	Pleopod exopod	9	9	8	7	6	6
10	6.1	Pleopod endopod	10			8	7	
(10)	6.3	Appendices internae	11	10	9	9	8	7
PL	6.3	Postlarva	12	11	10	10	9	8

who found pigment spots to be variable in position. Minor morphological details, which might serve as indices of species, were not discussed or figured by Faxon. It seems possible that he may have dealt with larvae of more than a single species, although Holthuis (1952) feels that the adults and, presumably, the first stage larvae described by Faxon were *P. vulgaris*.

The normality of larvae reared in the laboratory has been questioned by Gurney (1942) who believes that abnormal stages may be reared under artificial conditions. Gurney further states, however, that "extra stages," through which each individual need not pass in development, occur in nature. Numerous references to these extra stages are available (Faxon, 1879; Gurney and Lebour, 1941; Lebour, 1940).

Fraser (1936) and Boden (1950, 1951) defined stages or norms of variation in euphausid Furcilia larvae on the basis of the frequency of occurrence of all the forms encountered. As a result of these analyses it is possible to state that some of the forms are normal and some abnormal in a purely statistical sense. No treatment of the frequency of occurrence of variation in larvae of a decapod is available. It is not presently possible to state with certainty that any individual decapod larva is either normal or abnormal except on the basis of the course of its development. Defining normal development, however, presents great difficulties. MacDonald (1927) and Fraser found numerical predominance of certain of the euphausid Furcilia stages over others. It was assumed that certain of the stages may be skipped in development. This assumption was confirmed by Fraser in the laboratory. The normality of individual larvae and the course of larval development possibly depends upon extrinsic factors. Sandoz and Rogers (1944) found optimum temperature and salinity conditions for molting of *Callinectes* larvae and that the tempo of molting might be reduced by a sub-optimal diet. In view of the

lack of evidence to the contrary, the variation observed in the structure of larvae and the tempo of development in *Palaemonetes* is considered to be within the limits which may be considered normal for the species.

The apparent discrepancy in the proposed developmental sequence, shown by the unfolding of pereiopods 3 and 5 before the appearance of pleopods in some larvae and after the pleopod buds have been formed in others, may be a function of rate rather than sequence of development. Pereiopod 3 appears after the second molt and is followed by pereiopods 4 and 5. These may appear all at once in the third intermolt among rapidly developing larvae, or their appearance may be in separate intermolts. All the fourth intermolt larvae have five pairs of pereiopods of which the last three are rudimentary. Among rapidly developing larvae the pleopod buds appear in the fourth intermolt, before the last three pereiopods have unfolded. If pleopods do not appear in fourth, fifth or sixth intermolt larvae, pereiopods 3 and 5 will have become functional before the pleopod buds are first seen. Pleopods always follow pereiopods in appearance. The length of time or number of intermolts which intervene may permit some variation in the status of pereiopod unfolding at the time of the pleopod appearance.

The present concept of the crustacean larval stage has contributed to confusion regarding development. Most authors refer to each larval intermolt as a stage. Numbers are assigned to these stages which presume a knowledge of the molting history of the individual. Thus, a larva is assumed, on the basis of structure alone, to have molted a certain number of times and, presumably, to be of a certain age. The basis for this implication is an assumed norm of development on which reasonable doubt may be cast.

Development in arthropods is made to appear discontinuous by the inflexibility of the exoskeleton during the intermolt period. The morphology of the individual larva, which cannot change during the intermolt period, is determined by the extent of development completed at the time of the last molt. Fraser (1936) feels that, within limits, the time of molting may shift slightly backwards and forwards. This fluctuation, superimposed on a continuous process of development, was suggested as the cause of variation in euphausiid larvae. Heegaard (1953) has suggested variation in the rate of larval development in penaeids, but did not discuss molting. No variation in larvae is possible if either a causative or a casual relationship between molting and development exists so that each larval molt occurs at a precise time in development. The presence of larvae of the same molting history which vary widely in size and form argues in favor of the independence of the frequency of molting from the rate of larval development.

Arbitrary stages may be defined in crustacean development but should not be thought of as inflexible, natural steps through which each individual passes. The choice between few or many stages is possible. If, as has frequently been the case, each form found is described as a stage, then many individuals may skip certain stages in development. If few stages are defined, certain individuals may pass through what might be called extra stages during the course of normal development.

The inference that the variation in tempo of larval development is related to diet is inescapable. Trophic conditions may vary in nature during the breeding season of each species. At Beaufort, *Palaemonetes* larvae hatched in late April or May become part of a plankton community which is poor in total number of

organisms. A possible response to this sub-optimal condition might be prolonged larval life with a greater number of larval intermolts. Normal development of a decapod may itself vary according to the season of the year.

Summary

1. *Palaemonetes pugio* Holthuis and *Palaemonetes vulgaris* (Say) were reared in the laboratory from eggs through metamorphosis.

2. The larvae of these species are very nearly identical except for a pair of chromatophores found on abdominal sternite 2 of *P. pugio* but lacking from *P. vulgaris*. The sequence of development is the same for each species.

3. Individual larvae which were of the same age or which had molted the same number of times were not necessarily alike. Larvae of the same size, regardless of age or the number of molts completed, were alike. This discrepancy between age and development seems associated with the diet of the larvae.

4. The molting frequency of the larvae was independent of the rate of development.

5. Descriptions of a series of *P. pugio* larvae which illustrate the sequence of events in larval development are given. Some of these forms may be skipped.

6. The concept of the crustacean larval stage has assumed a constancy of development at variance with the facts observed in rearing experiments. The form of a larva alone may not be regarded as indicative of its age or previous molting history.

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THE RELATIONSHIP BETWEEN DIET AND LARVAL DEVELOPMENT OF PALAEMONETES [1, 2]

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Larval development of crustaceans consists, in its simplest form, of growth and the addition of somites and limbs. The primitive pattern has been obscured among higher crustaceans by the degree of development achieved by the embryo before hatching and by the magnitude of developmental change which may become evident after each larval molt. Gurney (1942) and most authors think of the mode of development for each species as fixed and regard the number and sequence of definitive larval stages as constant.

Variation in the number and form of larval intermolts of *Palaemonetes pugio* Holthuis and *Palaemonetes vulgaris* (Say) reared in the laboratory has been described by Broad (1957). The present paper is a consideration of the relationships between diet and survival, molting frequency, rate of larval development and the number and form of intermolts during development of these species.

The author is indebted to Professor C. G. Bookhout, under whose guidance this work was done. He also wishes to express his thanks to Dr. T. R. Rice, who kindly furnished stocks of all species of unicellular marine algae used.

Methods

Larvae dealt with were hatched in the laboratory from eggs carried by adult females and were reared through metamorphosis. Culture methods have already been discussed (Broad, 1957) and need not be repeated in detail.

Each larva was fed the same diet throughout its life, but several different foods were offered to different individuals. The diets differed from one another generally and specifically. There were five general diet categories: no food; unicellular marine algae; algae and non-living animal matter; non-living animal tissue alone; and living *Artemia* nauplii. Specific differences in diet were between the several combinations of the available foods in each general category.

Larvae which were not fed nevertheless had available whatever food might be obtained from the raw sea water in which they were reared. The form of the mouthparts of these larvae makes it extremely unlikely that particles not visible to the unaided eye could be utilized as food.

Some individuals were fed species or combinations of species of unicellular marine algae. These species were maintained in unialgal culture in the laboratory.

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[2] Part of a thesis submitted to the graduate faculty of Duke University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Clumps of cells which settle in older cultures were used as food. The algae available were from T. R. Rice's stocks of *Nitzschia closterium* and *Nitzschia* sp., (Bacillareae), *Chlamydomonas* sp.; *Thorocomonas* sp.; *Nannochloris* sp. (Chlorophyta); *Porphyridium* sp. (Rhodophyta) and *Pyramimonas* sp. (Chrysophyta).

Other larvae were fed non-living animal tisues alone or in combination with one or more of the algal species. Animal matter used was obtained largely from the plankton. Zooplankton, collected by net, was killed by immersion in distilled water to prevent the fortuitous inclusion of living larvae which might later be confused with individuals being reared. The general zooplankton (and possibly a few contaminating phytoplankton cells) was fed to some larvae. Others were fed on chaetognaths removed from the killed plankton. A few individuals were fed macerated gonad of the mud snail, *Nassarius obsoletus*.

The larvae usually hatched at night. The day on which free-swimming individuals were found was called the first day of larval life. Larvae and the bowls in which they were held were inspected daily. Only the presence of an exuvium was accepted as evidence of molting. Molts were considered to have occurred the day on which the cast exoskeleton was found. Uneaten food was removed and fresh food added at the time of daily inspection.

Results

Palaemonetes larvae were found to ingest almost any particulate matter with which they came in contact. There was no evidence of chemoreception or of selection of any type of food. Cannibalism was infrequently observed. In feeding, the zoea larvae grasp and hold objects with the maxillipeds while the maxillae and mandibles function as jaws. There was no indication of ability to obtain food by filtering.

Larvae fed diets which differed in general composition showed different rates of survival and development and molted at different frequencies, but those fed diets which differed only in specific composition did not. All the larvae are grouped for treatment of data into five general categories according to the diet received.

Diet and survival of larvae

Figures 1 and 2 show the per cent of mortality observed at each molt among *P. pugio* and *P. vulgaris* larvae fed various diets. Most of the deaths occurred at the time of molting, but some individuals were lost during the intermolt phase. These are included among the larvae which died at the time of the next molt. The per cent of larval mortality at molt N is that fraction of the total number of larvae which completed molt N-1 but did not survive molt N, and includes larvae lost during intermolt N. Loss of some intermolt larvae was due to removal of specimens for preservation or study, and the apparent mortality accordingly is biased upward when the total number of larvae involved is small or in the later molts.

Larvae which were not fed did not survive nor did those which were fed only phytoplankton cells. Sixty *P. pugio* larvae were starved. Forty-two of these survived one molt and two individuals molted twice, but none lived longer than 10 days. Among 80 *P. vulgaris* larvae which were not fed, none molted and all died within 5 days. Feeding any of a variety of unicellular marine algae did not seem

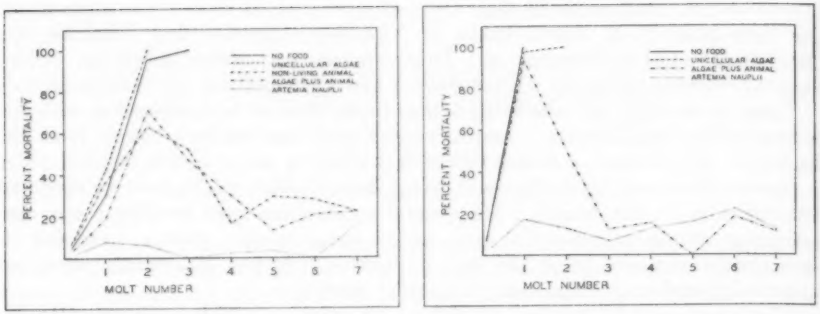

FIGURE 1. Mortality at each of the first seven molts among *Palaemonetes pugio* larvae fed various diets. Per cent mortality at each molt is based on the number of larvae which survived the last molt.

FIGURE 2. Mortality at each of the first seven molts among *Palaemonetes vulgaris* larvae fed various diets. Per cents computed as for *P. pugio* (Fig. 1).

to improve survival or molting in either 280 *P. pugio* or 100 *P. vulgaris* larvae. The survival of algae-fed larvae shown in Figures 1 and 2 closely approximates that of starved larvae.

Larvae that were fed foods of animal origin, either living or non-living, alone or in combination with algae, were able to survive through metamorphosis in the laboratory. The mortality of 608 *P. pugio* larvae that were fed diets of non-living animal matter, mostly zooplankton, combined with phytoplankton cells is shown by the dash-and-two-dots line in Figure 1. Sixteen of these individuals metamorphosed. The dash-and-dot line in Figure 1 shows the mortality of 732 *P. pugio* larvae that were fed non-living animal matter alone. In general the two curves are alike. Both reflect, in peaks at the second molt, the ability of this species to survive a single molt without food. Only 6 individuals metamorphosed on a diet of freshly killed zooplankton or other non-living animal matter. By far the best survival was shown by larvae fed living *Artemia* nauplii. The mortality of 100 of these is shown by the dotted line in Figure 1. Sixty-five individuals survived metamorphosis. Another 40 *P. pugio* larvae, for which it was not possible to determine daily the state of development of each individual, were fed living *Artemia* nauplii. Mortality at each molt of these individuals is not shown, but 33 metamorphosed.

The mortality of 667 *P. vulgaris* larvae that received a diet of non-living animal matter combined with algal cells is shown by the dash-and-two-dots line in Figure 2. The initially high mortality shown by starved larvae is also evident for these larvae, and contrasts with the early independence of available food shown by *P. pugio*. Six postlarvae survived metamorphosis. The dotted line in Figure 2 shows the mortality observed among 390 *P. vulgaris* larvae fed living *Artemia* nauplii. One hundred twenty-two of these metamorphosed in the laboratory.

Diet and the frequency of molting

Molting of *P. pugio* larvae is shown in Figure 3. The differences in molting frequencies between larvae which survived and most of those which did not on

each diet are insignificant, although those individuals which did not maintain a regular molting schedule did not survive. In general, the range of days during which specific molts occurred among larvae which lived is more restricted for the earlier than for the later molts. This most likely results from some variation in the molting frequencies of larvae fed similarly.

Since, except for the first two molts, there is little or no overlap in the means and standard deviations, or sometimes even the ranges, of corresponding molts by larvae fed different diets, the frequencies suggested by the diagram seem to be statistically separable. Average molting frequencies may be computed, although these become more or less meaningless since the interval between hatching and the first molt and that between the last larval molt and the molt of metamorphosis must be included. The interval between hatching and the first molt is usually somewhat longer than that between subsequent molts. The molt of metamorphosis, although not exactly comparable to other molts, is also included in the computations of average molting frequencies. Among larvae which survived metamorphosis on a diet of mixed plant and animal matter, the frequency of molting varied from one molt every 3.15 to 4.0 days with an average frequency of one molt every 3.70 days. Those larvae which survived on a diet of non-living animal matter alone molted once every 2.36 to 2.67 days with an average frequency of one molt every 2.51 days. Molting among larvae which survived metamorphosis on a diet of living *Artemia*

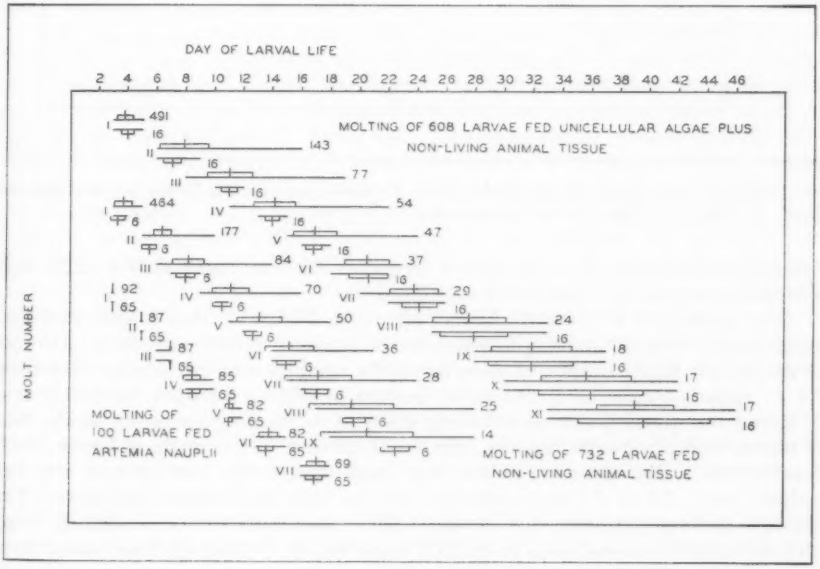
DAY OF LARVAL LIFE
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46
MOLT NUMBER
MOLTING OF 608 LARVAE FED UNICELLULAR ALGAE PLUS NON-LIVING ANIMAL TISSUE
MOLTING OF 100 LARVAE FED ARTEMIA NAUPLII
MOLTING OF 732 LARVAE FED NON-LIVING ANIMAL TISSUE

FIGURE 3. Frequency of the several molts among *Palaemonetes pugio* larvae fed three different diets. Number of each molt is given by Roman numbers. Upper line of each pair shows days during which molt occurred. Lower line gives days on which those larvae which completed metamorphosis molted. Means shown by vertical lines. Box on each side of mean is one standard deviation. Arabic numbers show sample size.

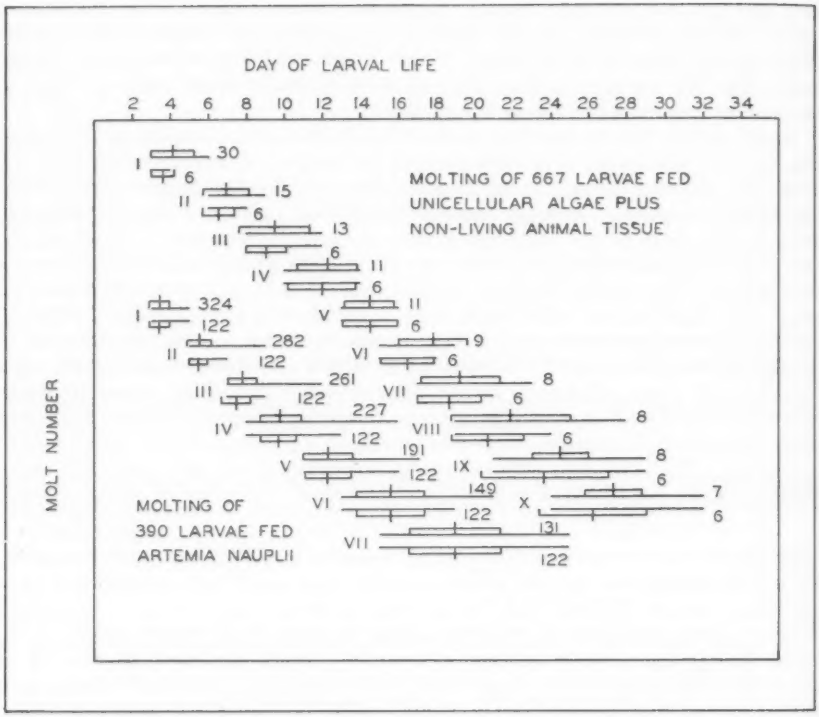
DAY OF LARVAL LIFE
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
MOLT NUMBER
MOLTING OF 667 LARVAE FED
UNICELLULAR ALGAE PLUS
NON-LIVING ANIMAL TISSUE
MOLTING OF
390 LARVAE FED
ARTEMIA NAUPLII

FIGURE 4. Frequency of each molt among *Palaemonetes vulgaris* larvae fed two different diets. See legend of Figure 4 for explanation.

nauplii was observed to occur with a frequency of once every 2.30 to 2.50 days with an average of one molt every 2.43 days.

The molting of *P. vulgaris* larvae, shown in Figure 4, shows little statistical separateness between molting frequencies of larvae fed different diets. Because of the initially high mortality of these larvae the samples are even smaller than those for *P. pugio*. Fourth and fifth molts seem to be distinct between the two groups of larvae, but, possibly due to a slowing-down of the molting frequency as the time of metamorphosis approaches, the later molts are not. Among those larvae which survived on a diet of mixed animal and plant matter, the molting rate was one ecdysis every 2.4 to 3.1 days with an average of a molt every 2.62 days. The average molting frequency for larvae which metamorphosed on a diet of living *Artemia* nauplii was one molt every 2.67 days, but the molting of these varied from one molt every 2.1 to 3.6 days.

Diet and the rate of larval development

An approximation of the rate of larval development may be obtained from the number of days between hatching and metamorphosis. This, however, is not

completely satisfactory since metamorphosis need not always occur at what appears from morphological criteria to be the end of larval development. Thus, Faxon (1879) found "last stage" *P. vulgaris* larvae which molted in the laboratory gave rise to other "last stage" larvae morphologically indistinguishable from their previous form. The larvae of both *P. pugio* and *P. vulgaris* reared in the present study sometimes passed through two or more identical intermolts at the end of development and before metamorphosis. At this time, depending upon other conditions not presently understood, the larva may metamorphose, as most of them do, or possibly may remain larval for some time.

P. pugio larvae fed a diet of mixed animal and plant matter metamorphosed from 29 to 49 days after hatching. Most of the larvae metamorphosed at the eleventh or twelfth molt, but a few metamorphosed as early as the ninth or tenth molt, or as late as the thirteenth molt. Larvae of this species fed a diet of non-living animal matter alone metamorphosed from the nineteenth to the twenty-eighth day after hatching and at the eighth, ninth, tenth or eleventh molt. Those individuals fed a diet of living *Artemia* nauplii metamorphosed from 17 to 21 days after hatching, usually at the seventh, but sometimes at the eighth molt.

P. vulgaris larvae fed a diet of mixed animal and plant matter metamorphosed from 24 to 34 days after hatching. The molt of metamorphosis was most often the tenth, but two individuals metamorphosed at the eleventh and thirteenth molts. Those larvae fed a diet of living *Artemia* nauplii metamorphosed from 14 to 30 days after hatching, usually at the seventh molt. Metamorphosis was also noted at the sixth, eighth, ninth and tenth molts.

A second approximation of the rate of development might be obtained by comparing the structure of larvae to age and molting history. In spite of the variation noted in duration and tempo, the sequence of development was the same for all larvae and for both species. Except for differences in color patterns which are specific, all newly-hatched *P. pugio* and *P. vulgaris* larvae were essentially alike. For the present purposes this first zoea larva may be characterized by the presence of all head appendages, three pairs of functional maxillipeds, two rudimentary pereiopods, sessile eyes, a fan-shaped telson and a lack of spines. After a single molt all larvae acquire stalked eyes, spines on the carapace and abdomen, and functional first pereiopods. Until after the second molt variation in form is almost non-existent, but, from the second molt to the end of larval development, there may be wide variation in the form of larvae of the same age or molting history. A table which summarizes the relationship between intermolt number and form of larvae fed variously has previously been presented, and the morphology of the several developmental steps or stages has been discussed in detail (Broad, 1957). For the present purpose it suffices to reiterate that the number of intermolts in development of either species dealt with may vary, but that the sequence of events in development does not. Approximate ages of intermolts discussed below may be obtained from Figures 3 and 4.

Following the second molt, third intermolt, or third zoea larvae show variation in the number of pereiopod rudiments. Those individuals fed *Artemia* had rudiments of pereiopods 3, 4 and 5 while those fed other diets lacked pereiopods 4 and 5. Great variation was evident among fourth intermolt larvae. Those individuals fed *Artemia* bore rudiments of the pleopods but none of the others did. Among larvae fed non-living animal foods alone, pleopod buds first appeared in the fifth or

sixth intermolt and not until the seventh intermolt in larvae fed diets which combined algae with animal matter. The intervening intermolts, 4, 5 and 6, differed from one another in the development of pereiopods.

Fifth intermolt larvae which had been fed *Artemia* had pleopod rudiments and chelae. The pleopod buds of fifth or sixth larval intermolts fed non-living animal tissue alone became rudiments after a single molt, but chelae did not appear until after two molts. Some of the eighth larvae fed algae and animal matter bore chelae, but others had none.

The pleopods of all sixth intermolt larvae which had been fed *Artemia* bore setose exopods. These were first evident in seventh or eighth intermolts which had received non-living animal food and in ninth zoea larvae which had been fed algae plus non-living animal foods.

The final larval form has been characterized by both setose endopods and *appendices internae* on pleopods. Most larvae fed *Artemia* nauplii achieved this final form in the seventh intermolt, although some individuals required two steps after the sixth zoea for *appendices internae* to appear. Metamorphosis occurred at the seventh or eighth molt. *Appendices internae* made their appearance in ninth intermolt larvae fed non-living animal matter, and metamorphosis occurred at the tenth molt. Among larvae fed algae and animal matter, the final larval form was reached in the tenth, eleventh, or even sometimes the twelfth intermolt. Metamorphosis usually occurred at the following molt.

Discussion

Since survival of larvae fed algal diets alone did not differ from that of zoeae which were not fed, it would seem that the algal species available had no value as food for either *P. pugio* or *P. vulgaris* larvae. In order to survive, the larvae must find some particulate food, probably animal in nature.

A physiological distinction between *P. pugio* and *P. vulgaris* is possible in the ability of the former species to molt once and survive up to ten days without food while the latter neither molts nor lives longer than five days without food. Speculation regarding the survival value which might be associated with this relatively greater independence of trophic conditions, though interesting, is futile in view of the presently limited knowledge of the geographic and ecological distribution of the species of *Palaemonetes*. The ability of either species to adjust development to external conditions and the rather indefinite time of metamorphosis are both of positive survival value.

It is possible that the varying rates of survival, molting and development noted among larvae reared in the laboratory may be due to differences in the total quantity rather than in the quality of food available. Two factors limited the amount of non-living animal tissue available to larvae fed this diet. In order to retard fouling of the water in the bowls, the total quantity of food added daily had to be kept within limits. Since food was always left, the quantity was at first thought to be sufficient. Non-motile food, however, sank to the bottom of the bowls. The larvae swam near the surface. It has already been stated that contact between zoea and food seemed to be the result of chance encounter rather than active search on the part of the larva. The low probability of encounter between larvae swimming near the surface and food lying on the bottom might account for the uneaten food left each day.

If clumps of algal cells were added to the diet, a third limiting factor is also added. Larvae fed algae actually ingested the material offered. The red, green or brown color of the cells could be seen in the gut and feces of the larvae. Since the algal species used have been shown to be of no nutritive value, it seems possible that, where algae plus another food is offered, the ingestion of the nutritively inert material may restrict the intake of other foods which can be digested and utilized.

Ne restrictions were placed on the total number of *Artemia* nauplii fed. The nauplii swam near the surface with the zoea larvae. The probability of encounter between larva and food was greatest when the diet consisted of living animals.

It has been suggested that intraspecific variation in crustacean larvae may arise from extrinsic causes (Broad, 1957). Sandoz and Rogers (1944) found that poorly nourished *Callinectes* zoeae molted only after a relatively long period and were smaller than other larvae which had received more food. Templeman (1936a, 1936b) found reducing the amount of food given *Homarus* larvae lengthened the intermolt period and sometimes resulted in the production of an "extra" larval intermolt. The present data suggest that the amount of food available to *Palaemonetes* during its larval life may affect both the rate of development and the frequency of ecdysis. The independence of these variables is suggested by the differences in the magnitude of response to sub-optimal feeding. Although the longest regular interval between molts was only 1.9 times the least, the duration of larval life was extended 3.5 times over the most rapid successful development.

Heegaard (1953) has suggested that different developmental rates among decapod larvae may be caused by "external as well as internal" factors. The amount of available food may be an external factor which affects both the rate of development and, independently, the frequency of molting. Variation in the form of larvae under these conditions might be restricted only by the morphological limitations of species. If norms of developmental stages exist among decapods, as Fraser (1936) has found among Euphausids, constancy of environment might be considered of prime importance in their establishment. Extra or abnormal larval stages found in nature or in the laboratory as well as stages skipped in development may reflect an adaptibility to environmental variation during development.

Summary

1. Larvae of *Palaemonetes pugio* Holthuis and *Palaemonetes vulgaris* (Say) reared in the laboratory showed differences in survival, frequency of molting and rate of development which may be associated with the amount of food available.

2. Larvae were unable to survive if fed diets of either single species or combinations of species of several unicellular marine algae or if not fed. Starved *P. pugio* larvae were able to survive one molt without food but starved *P. vulgaris* larvae died without molting.

3. Larvae of both species lived through metamorphosis if fed a diet which included animal tissue. The best survival was obtained by feeding living *Artemia* nauplii.

4. The frequency of molting, the duration of larval life and the number of larval intermolts in the development of *P. pugio* and *P. vulgaris* vary according to the quantity of food available. The frequency of molting and the rate of development are suppressed by a reduction in intake of food.

5. Variation in molting frequency independent of the rate of development makes possible variation in the form and number of larval intermolts.

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X-RAY EXPERIMENTS WITH MOLGULA MANHATTENSIS: ADULT SENSITIVITY AND INDUCED ZYGOTIC LETHALITY

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Sessile organisms which pump large quantities of sea water through their bodies suggest themselves as ideal material in which to study biological effects of radioactive contaminants. Because of their relationship to the chordates, ascidians have particular attraction. Furthermore, many are functional hermaphrodites providing both sperm and eggs for simultaneous irradiation. *Molgula manhattensis* has the additional advantage of self-fertility so that crosses using gametes from the same individual can be made, as well as outcrosses.

In the absence of radiobiological information on Molgula, the present x-ray experiments were performed rather than employing isotopes with their more difficult dosimetry. Isotope experiments may be performed in the future after a more complete knowledge of what may be expected from external radiations is at hand.

Materials and Methods

Preliminary experiments were performed during the summer of 1955 with the assistance of Robert L. Sullivan. Specimens of Molgula were collected for us by personnel of the M. B. L. Supply Department. Irradiation was delivered by the M. B. L. generator in "A" position at 6000 r per minute (187 KV; 28 ma; filtration = 0.2 mm. Cu). To achieve massive doses without overheating, the animals were immersed in sea water within an inner chamber, and crushed ice was packed into the space between it and the outer wall of the plastic container. After irradiation, animals were kept in individual glass jars (50 × 80 mm.) in running sea water.

Failure to contract in response to prodding with a blunt instrument was the criterion for death. An additional check on presumptive death was obtained by holding such animals until post-mortem flaccidity and degeneration became obvious. The results indicated that adults would be adequately resistant to whole-body irradiation which would allow the full range of dosages required for a genetic experiment on induced dominant lethality.

In 1956 animals of adult size were obtained both through the M. B. L. Supply Department and by personal collection. The latter procedure seemed necessary when it was discovered that masses of Molgula held in the Eel Pond "live-cars" and in laboratory aquaria did not consistently exhibit gonads with mature gametes. The adequacy of the supply of eggs and sperm can be determined by examination with a low power microscope, usually after peeling off the test, always after removal of a portion of the mantle.

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Adults were irradiated in position "B" at 2500 r per minute, again using the Woods Hole machine, the Coolidge tubes this time not as close to the animals.

Eggs were obtained by suction applied to a capillary pipette inserted into the lumen of an ovary. They were expelled into a stender dish of sea water. Immediately after withdrawing eggs from both ovaries, sperm suspensions were prepared by macerating a portion of each testis in a separate dish of sea water (3–5 cc.).

Eggs from each ovary were divided into three groups: (1) a control as a check against accidental fertilization during removal, (2) a sample which was fertilized with sperm from the adjacent testis, and (3) a sample which was cross-fertilized with sperm from another Molgula. Sixty-millimeter flat stender dishes, containing one inch of sea water, were used. They were placed on the sea table with their bases in running water. Fifteen hours later, tadpole development was scored using a stereoscopic dissecting-type (48 ×) microscope. Immediately after completion of these observations, the degree of cleavage for 100 objects was scored using a compound microscope (16 mm. objective, 10 × ocular).

During July and the first half of August, pair-mating experiments were set up daily to obtain simultaneous data (a) for eggs from an irradiated animal fertilized by sperm from an untreated one, (b) for the reciprocal cross of treated sperm to untreated eggs, (c) from selfing the irradiated animal, and (d) selfing the non-irradiated member of the pair. The goal was to obtain data from at least five pairs of crosses for each of five chosen doses.

T. H. Morgan's 1942 paper led us to expect a technical difference between the two ovaries in difficulty of removing unfertilized eggs. Therefore we kept separate controls and made separate crosses for each side of each animal. However, upon analysis of results, no significant differences between sides were demonstrable and the data for the two sides of each animal serve merely as replications.

From August 13 through August 18 the daily plan of experiment was modified. On each day, five or more adult animals were simultaneously exposed to one of the five chosen doses. The gametes obtained from five treated animals were mixed to provide outcross data when both sperm and eggs were treated. At least five samples (averaging 800 objects/sample) were scored for each mass fertilization. On August 15 a mass outcross control was obtained.

From August 20 through August 24, five animals were treated simultaneously at each of the five doses and selfed to obtain more information about the variability between material from different treated animals.

From August 27 to September 1, miscellaneous experiments were set up: crosses of three different animals, each given a different dose, to a single untreated animal, and selfing crosses in which several doses were investigated each day.

Results

Lethality of adults

An exploratory experiment indicated that the critical dose of x-ray for the adult organism lay between 36,000 r and 60,000 r. This involved observations on 72 sea-squirts, 8 of which were controls, along with 8 samples of 8 animals given doses graded between 1000 r and 120,000 r.

Two subsequent experiments of 40 each were set up with controls and samples irradiated at the following doses: 36,000 r, 42,000 r, 48,000 r, 54,000 r and

60,000 r. In both the control and 36,000 r groups, individual animals were still alive more than a month after the date of irradiation. Also, although most of the animals given 42,000 r died during the first week, one lived almost a month. At higher doses no animals survived the fourth day, with the average time of death 2.4 days after treatment. This places the lethal dose between 42 and 48 kiloroentgens. This is considerably less than the radiation required to kill adult insects (Sullivan and Grosch, 1953), brine shrimp (Grosch and Erdman, 1955) and vegetative microorganisms (Bacq and Alexander, 1955). On the other hand, such amounts of radiation are many times that required to kill mammals.

Induced zygotic lethality

A summary of the relative proportion of tadpoles obtained among the ova and zygotes studied is given in Figure 1. Consistently fewer swimming tadpoles emerged than developed to tadpole morphology. Furthermore, curves for data from irradiated eggs tend to lie below those representing sperm. This trend becomes statistically significant at higher doses and it should be re-emphasized that the data come from paired matings.

With self-fertilization, involving sperm and eggs from irradiated sea-squirts, the developmental yield is strikingly decreased (solid line, "Both Treated"). Along with the curve for selfing data, results from mass outcross experiments are shown by the broken line in Figure 1. With one exception, points obtained by calculating the mean are nearly identical whether selfing or outcrossing has been the procedure, provided both sperm and eggs are from irradiated animals. The standard errors omitted from the outcross curve in order not to further complicate Figure 1 are less than 2%.

An additional group of data obtained from selfing five animals irradiated simultaneously at each dose also gives a curve similar to the solid line at the lower doses and identical with it at the three higher doses. Therefore it is not shown here.

Since it has been corroborated by three separate sets of experiments, the curve above 5,000 r is a good representation of expectation when both sperm and eggs come from gonads irradiated *in situ*. Furthermore, this "Both-Treated" curve is predictable on the basis of one of the laws of probability: when two events are independent, the probability that both will occur simultaneously is the product of their separate probabilities. Thus, multiplying survival when sperm are x-rayed by survival when eggs are x-rayed we obtain the following:

$$\begin{aligned}
.33 \times .075 &= .0248 = 2.5\% \text{ for } 20{,}000\text{ r} \\
.34 \times .185 &= .0629 = 6.3\% \text{ for } 15{,}000\text{ r} \\
.45 \times .43 &= .1935 = 19.4\% \text{ for } 10{,}000\text{ r} \\
.545 \times .51 &= .2779 = 27.8\% \text{ for } 5{,}000\text{ r}
\end{aligned}$$

These values calculated from Figure 1 data for the five higher doses are all within the range of one standard error from the "Both-Treated" curve shown in Figure 1. The theoretical value for 1000 r, 24.6% (.53 × .465), is lower than the "selfing" value obtained in pair experiments but falls within the range found in outcross experiments when both sperm and eggs are from treated animals.

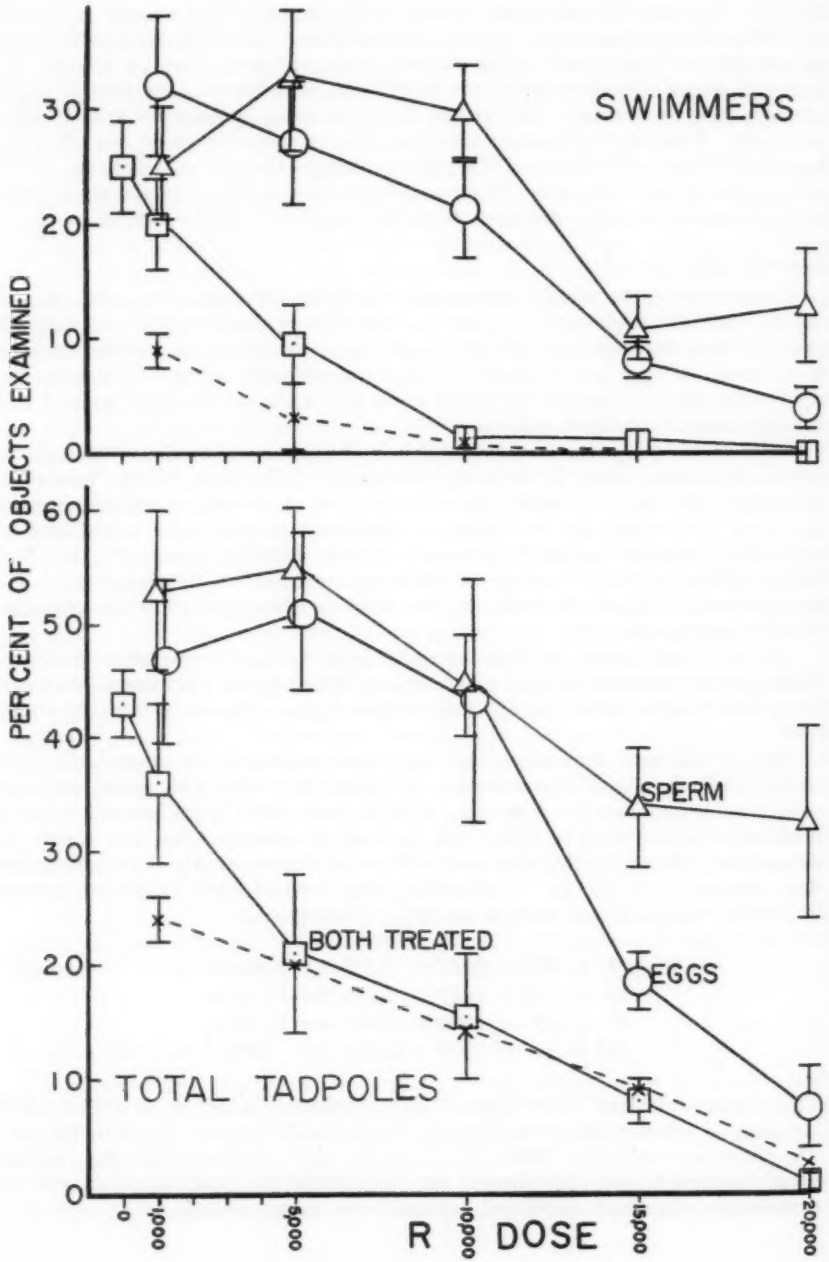
SWIMMERS
PER CENT OF OBJECTS EXAMINED
30
20
10
0
60
50
40
30
20
10
0
SPERM
BOTH TREATED
EGGS
TOTAL TADPOLES
0
5000
10000
15000
20000
R DOSE

TABLE I

Development in the residuum of eggs and zygotes, tadpoles having been counted. The scoring was done on 100 objects in each case. Values are given in per cent, representing failure to develop to tadpole morphology (means and standard errors)

X-ray dose in r	Both gametes treated			Sperm treated	Eggs treated
	Mass outcross	Self-cross 5 simultaneously	Self-cross pair experiments		
0	99.5 ± 0.5		45.8 ± 11.9		
1,000	84.3 ± 3.3	76.5 ± 0.5	54.2 ± 10.8	74.6 ± 6.3	63.8 ± 7.9
5,000	90.5 ± 2.0	72.2 ± 7.4	35.4 ± 12.9	55.4 ± 3.4	57.4 ± 9.8
10,000	89.9 ± 4.7	93.6 ± 2.9	59.2 ± 12.2	65.2 ± 5.6	64.8 ± 12.6
15,000	91.2 ± 1.2	79.8 ± 12.7	70.5 ± 8.4	65.9 ± 5.3	75.0 ± 6.8
20,000	86.1 ± 1.6		36.2 ± 10.2	76.0 ± 5.0	50.4 ± 11.7

No attempt was made to obtain a quantitative record of morphological aberration. However, as an indication of developmental difficulty, thickened, bent and misshapen tails were typical for tadpoles in experiments above 10,000 r.

Cleavage data

In percentages, Table I presents a summary of results when objects other than tadpoles were scored. These are the averages from five or more experiments, in each of which 100 objects were carefully examined. Because of the time required and technical difficulty it is not feasible to examine all of the residuum. In order to understand Table I it must be realized that when few tadpoles develop from a group of eggs there are many undeveloped forms, and vice versa. Accordingly, since the amount of residuum varies, similar percentages with different doses may actually reflect differences. A conversion of the tabulated percentage values into numerical values, such as those plotted in Figure 2, helps to visualize the situation. The average total of eggs per stender dish, 800, is used as a common basis for presentation. The pertinent percentage of tadpoles (see Fig. 1) is subtracted. The applicable cleavage percentage (Table I) of the remainder gives the relative number of embryos plotted in Figure 2.

All embryos considered in Table I and Figure 2 appeared to be in the late gastrula or neurula stages, although at the two highest doses, structure was very disorganized. Presumably if embryos were able to begin development they could continue to such stages before facing an insurmountable developmental crisis. Extremely few embryos were found halted in an early cleavage stage. During the whole summer, when nearly 150,000 examinations were made, only 18 early cleavage types were seen in selfing experiments, and 17 in outcrosses—exceptional individuals making up only 0.02%.

As might be expected, the number of gastrulae and neurulae increased as the number of tadpoles decreased at higher doses. Although this general trend is clear, unidentified sources of variability complicate the picture in selfing and pair-

FIGURE 1. The relative proportions of tadpoles developing from gametes obtained from adult specimens of Molgula after irradiation. Results are contrasted when either or both types of gametes come from x-rayed parents.

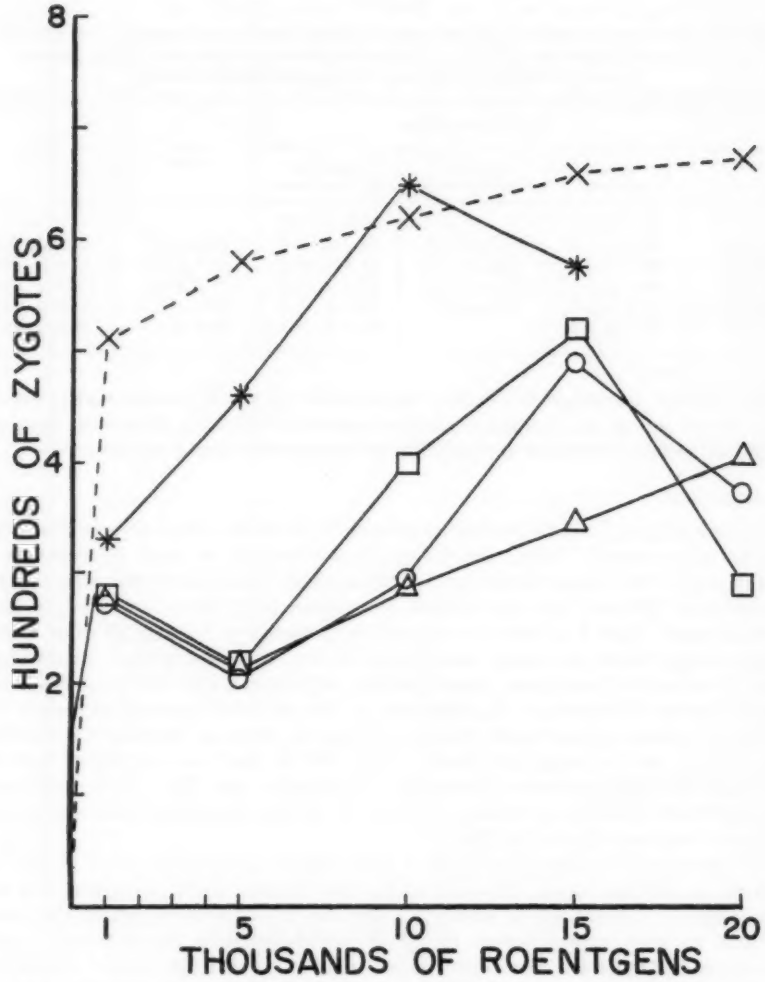

FIGURE 2. Post-cleavage zygotes which failed to develop to the tadpole stage. Results have been put on a common basis by calculations from the average total of eggs per sample, 800. Triangles indicate that the sperm were from treated animals; circles, eggs. Squares represent selfed eggs and sperm from treated animals in pair experiments; asterisks, data from 5 irradiated animals selfed on the same day. X's indicate outcross data, both parents x-rayed. Squares, circles and triangles represent results from pair experiments.

mating experiments. When plotted as in Figure 2, zigzag lines are obtained. Furthermore, in the latter experiments, results when both gametes came from treated parents are not greatly different from those obtained when only one of the

types of gametes has been exposed to radiation. Indeed, even after 5000 r, all three pair mating values were nearly identical. Therefore some general aspect of fertilization common to the experiments should be sought. The higher average values obtained after simultaneously selfing five animals given the identical dose of radiation are consistent with such a view.

Discussion

Relative radiosensitivity of sperm and eggs

In Molgula, relative radiosensitivity of eggs instead of sperm is an interesting parallel to Rugh's (1953) similar findings with the clam Spisula, although his crosses involved gametes which were irradiated after extraction from the parent. Such results are not expected, either on the basis of Henshaw's type of delayed cell division or that of chromosomal-gene effects. Sperm have been found more sensitive on either count.

Mavor and De Forest (1924), who irradiated Arbacia gametes, found that samples scored two days later showed development ranging from gastrula stages to well-formed plutei. The retardation in development was greatest in those larvae developed from x-rayed sperm and increased with dose. Subsequently, Henshaw (1940) explained this phenomenon by a demonstration that radiation-delayed first cleavage is reflected in later development. Furthermore, the apparent difference in sensitivity was due to partial recovery occurring in the eggs prior to fertilization.

Judging from insect experiments, dominant lethals are more readily induced in sperm. In fact, P. W. Whiting (1938) found that at dosages of about 10,000 r, practically every Habrobracon sperm contained at least one dominant lethal. Certain of his experiments more closely resemble the conditions of the present experiment than any other investigations we have been able to find in the literature. In these experiments, female wasps mated before treatment contained both sperm and eggs when irradiated. Comparisons with data from females mated after irradiation, and with parthenogenetic data, indicated dominant lethals to be more readily induced in sperm than even recessive lethals in the eggs. In a definitive Habrobracon paper, Heidenthal (1945) constructed the dominant lethal mutation curve for doses up to the asymptote and demonstrated that those secured for Drosophila (Sonnenblick, 1940; Demerec and Fano, 1944) were quite similar. An even steeper curve has been reported for Mellitobia (Kerschner, 1946). Muller and the Valencias (1949) have since presented Drosophila data which indicate that presumptive deficiencies are far less abundant if eggs are irradiated.

Nucleic acid or its cycle is implicated in both the Arbacia cleavage delay experiments and the dominant lethal experiments with insects. Although usually discussed separately, it seems possible that both types of damage may be reflected in the results obtained by scoring development at a specified time. However, it has been shown that neither phenomenon completely explains the present results. Perhaps a third and somewhat different aspect of cell division—other than chromosomal—is involved. The material may need to be considered from the standpoint of the general physiologist who studies the stimulus for initiating the process of cell division (Heilbrunn, 1955; Heilbrunn and Wilson, 1955; Rieser, 1955).

Especially provocative is an earlier Arbacia paper (Heilbrunn and Young, 1935), which shows that irradiation in the presence of ovarian tissue produced a

considerably greater division delay than irradiation of eggs in sea water alone or in concentrated suspension. At least, sperm inactivation can be ruled out. This requires doses in excess of 100,000 r in marine forms as well as insects (Maxwell, 1938; Henshaw, 1940; Rugh, 1953).

Self-sterility

Another aspect of Molgula investigations which may turn out to be a problem in physiology rather than genetics is fertility-sterility. A range from perfect self-fertility to absolute self-sterility has attracted geneticists to ascidians from the early days of genetics research (Morgan, 1904). However, although T. H. Morgan himself devoted considerable attention to the problem, including experiments with Molgula (1942), neither the genetic basis nor the physiological mechanisms have been completely elucidated.

Observations during the present experiments revise the Molgula picture. Self-incompatibility is not as extensive as previously believed, provided (1) that organisms with undeveloped or senile (degenerate?) gonads are not used, and (2) that no strong chemical cleaning solution or detergent is employed in cleaning glassware. In all our experiments in which these criteria were met, Molgula adults were self-fertile. The influence of a chemical agent was demonstrated dramatically one day when Alcanox had been used on the glassware. In spite of repeated water washings, as is the standard procedure in analytical chemistry, and over-night drying, no development occurred in selfing and only about 5% development in outcrosses. Ordinarily in outcrosses by far the great majority of eggs are fertilized and cleave. Although no details are available, at least one of Morgan's Molgula experiments bears a resemblance to this exceptional one of ours. He recorded a case in which no eggs selfed and only two out of a large number of eggs cleaved when cross-fertilized. Perhaps hot water is the only safe cleaning agent, although the junior author feels that dilute HCl rinses do much to offset the Alcanox type of hazard.

Summary

1. The lethal dose of x-rays for adult specimens of Molgula is placed in the neighborhood of 45,000 r (delivered at a rate of 6000 r/minute).

2. Radiation damage to gametes from irradiated adults can be measured in terms of tadpoles, unhatched or swimming. Eggs proved more sensitive than sperm. Curves when both gametes come from irradiated parents were similar no matter how obtained, pair matings or group matings, selfed or outcrossed. In the latter curves, 10,000 r is about the limit for swimmers, and 20,000 r for tadpole development (rate, 2500 r/minute).

3. The cleavage score for 100 objects residual to developed tadpoles did not provide a regular, clear-cut picture of radiation damage. It is suspected that uninvestigated features of fertilization physiology cloud the issue.

4. Self-incompatibility in Molgula is not as extensive as previously believed. The condition of the gonads must be considered and chemical cleaning solutions should be avoided.

5. It is concluded that the physiology of spindle formation rather than that of nucleination or chromosomal continuity may be a most important aspect of results like the present.

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ESTROGENS IN MARINE INVERTEBRATES

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Material with estrogenic activity demonstrable by bioassay in rodents has been found in marine invertebrate tissues. Steidle (1930), using a mouse bioassay, found traces of estrogens in a sea urchin, *Echinus miliaris,* three molluscs, *Aplysia, Octopus* and *Eledone,* as well as in certain worms and arthropods. Similarly Schwerdtfeger (1932) found estrogens in a sea anemone, *Actinia aquina,* but none in the mollusc *Chiton marginatus.* Donahue (1940) reported small amounts of estrogenically active material to be present in extracts of the Bermuda urchin, *Lytechinus variegatus,* the reef urchin, *Echinometria,* a holothurian, *Stichopus mobii,* and a lobster, *Palinurus argus.* More recently, this same author (Donahue, 1948) made extracts of the shed eggs of another lobster, *Homarus americanus,* purified them by solvent partition, and made estrogen analyses by a fluorometric method. In this way he found 100 international units of estrogen per 100 gm. eggs.

Gordon and Villee (1956) have described an enzymatic assay for estrogens, which is as sensitive as the fluorometric methods now available. Their assay depends upon the fact that human placenta contains a DPN (diphosphopyridine nucleotide)-linked isocitric dehydrogenase which catalyzes the reaction.

$$\text{isocitrate} + \text{DPN} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{DPNH} + CO_2$$

and which is specifically activated by certain natural estrogens. In a limited range the degree of enzyme activation is a function of the amount of hormone present. Since they measured the appearance of reduced DPN spectrophotometrically, it was essential that the material being assayed give a clear solution, and for that reason they found it impossible to analyze blood and tissue extracts. The progress of the reaction can also be measured chemically, by analyzing the reaction mixture after incubation for total keto-acids produced, and in this way relatively impure tissue extracts can be assayed. Such assays done on the ovaries of a number of marine invertebrates are reported here.

Methods

Invertebrate tissues were extracted for assay by the procedure used by Folch *et al.* (1954) for preparing total lipide extracts. The tissue was removed from the animal, blotted gently with filter paper, weighed accurately and homogenized in a Waring blendor containing 20 ml. 2:1 chloroform:methanol per gram of tissue. The homogenate was filtered and the residue discarded. The filtrate was washed

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once with 0.2 volume of distilled water and once with 0.2 volume of 0.01% aqueous calcium chloride which had previously been equilibrated with 2:1 chloroform:methanol. The extract was evaporated to dryness at room temperature under a gentle stream of clean air. The residue was taken up in ethanol for analysis and if, after thorough mixing, the solids did not go completely into solution, they were allowed to settle and the clear alcoholic extract was used for assay.

A 20% w/v homogenate of term human placenta was made in ice-cold 0.25 *M* sucrose, and centrifuged for 10 minutes at 600 G to remove cell nuclei and debris. The supernatant was then centrifuged at 57,000 G for 60 minutes to remove cellular particulate matter (Villee, 1955). The supernatant from the latter centrifugation contains the soluble matter of the cell, including the DPN-linked isocitric dehydrogenase. The enzyme-catalyzed reaction was carried out in 20-ml. beakers in a Dubnoff incubator at 37°, and was allowed to proceed for one hour. Each reaction vessel contained one ml. of the placental enzyme preparation; one ml. buffer containing 30 micromoles K^+, 10 micromoles Mg^{++}, 20 micromoles phosphate, and 20 micromoles Cl^-, adjusted to pH 7.3; 0.9 ml. water containing 0.75 micromole DPN and 3 micromoles cis-aconitate; and 0.1 ml. ethanol in which the standard or unknown solution was dissolved. The reaction was started by adding the DPN. Crystalline estradiol-17β was used as a standard. Analyses of the reaction mixture for total keto-acid production were made by a modification of the method of Friedemann and Haugen (1943). The amount of keto-acid produced in vessels containing all components except estradiol was used as a blank correction for the standard, and that produced in vessels containing all components except DPN was used as a blank correction for the unknowns. Total nitrogen analyses of the enzyme preparation were made by a micro-Kjeldahl procedure. The keto-acid analysis results were calculated in micromoles keto-acid produced per milligram nitrogen per hour. Duplicate reaction vessels were incubated and analyzed for each of the standards and unknowns in each assay.

Results

Separate standards were assayed with each set of unknowns, and the average results from 12 sets of standards are plotted in Figure 1. The least squares curve for these points is also shown. There is clearly a linear relationship between the logarithm of the amount of estradiol added and the amount of keto-acid produced over the range from 0.05 to 0.25 microgram estradiol per vessel. The index of precision, (λ), for this curve is 0.3 (Bliss, 1944). Recoveries of estradiol added to tissue before extraction averaged 99%.

The amount of estrogen in each unknown was calculated from a simultaneously determined standard curve for the same placental enzyme preparation. Each tissue extract was assayed with at least three different enzyme preparations from three different placentas. Of the tissues extracted, only the ovaries of *Mactra* (*Spisula*) *solidissima* contained significant amounts of estrogenic material, 1.1 $\pm$ 0.4 (mean $\pm$ standard error) milligrams estradiol equivalents per kilogram of fresh tissue. The other tissues, including the ovaries of *Asterias forbesi, Arbacia punctulata, Strongylocentrotus droebachiensis, Loligo pealei, Busycon canaliculatum, Carcinides maenas, Homarus americanus,* and *Limulus polyphemus,* the whole tissue of *Microciona prolifera* and the liver of *Homarus americanus* all contained less than 50 micrograms estradiol equivalents per kilogram of fresh tissue.

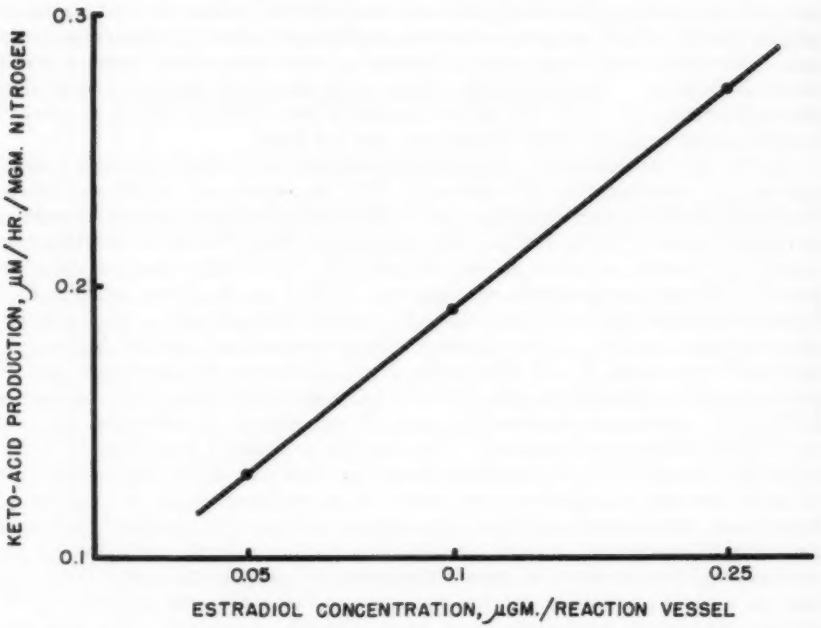

FIGURE 1. Keto-acid production by placental isocitric dehydrogenase as a function of estradiol-17β concentration.

DISCUSSION

The results were calculated in terms of estradiol equivalents, since that compound was used as a standard. It has been shown (Villee and Gordon, 1956) that estrone, equilenin and equilin are as effective as estradiol in stimulating the enzyme, while a variety of other steroids and synthetic compounds do not affect the enzyme unless present in very large quantities. It is thus likely that the material present in *Mactra* ovary is one of these four compounds.

The minimum amount of estrogen that can be detected in this assay is about 0.01 microgram of estradiol, and the tissue concentration that can be accurately determined depends, of course, on the amount of tissue extracted. With samples of tissue of about 10 grams, the analytical blanks are not unduly large and this magnitude of sample was used in these experiments. In all the tissues examined except *Mactra* ovary, the results of the assays indicated the presence of small amounts of active material, confirming the experience of earlier workers. The amounts are probably much smaller than the upper limit of 50 micrograms per kilogram which the present results make certain, but it would be necessary to extract larger quantities of tissue to make accurate estimates of the true amount present.

The precision of which this assay method is capable depends on the same factors as any other bioassay, the index of precision of the standard curve and the number

of replicates which are made. The former is satisfactory and the effort expended in making many replicates by this method is less than with many other forms of bioassay. Even with the small number of assays that were done in the present work on the *Mactra* ovary extracts, one can be quite confident that this tissue contains a very large amount of estrogenic material, presumably estradiol or a closely related compound. One milligram per kilogram is ten times as much as the maximum previously reported (lobster eggs) for any species. Estradiol added *in vitro* has no effect on the metabolism of *Mactra* ovary (Hagerman, 1956) and apparently no physiologic effects of estrogens on molluscs have been described. The material may be present as an evolutionary freak, similar to the occurrence of uric acid as an excretory product in the Dalmatian coach hound, or may have some important physiologic function in this mollusc. Further speculation should await the isolation and complete chemical characterization of the material present in *Mactra*.

Summary

1. An enzymatic method of assay for estrogens suitable for use with crude tissue extracts is described.

2. Of a variety of marine invertebrates examined, only the ovaries of the mollusc, *Mactra* (*Spisula*) *solidissima*, contained appreciable amounts of estrogenic material.

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THE EFFECTS OF X-IRRADIATION ON THE FERTILIZED EGGS OF THE ANNELID, CHAETOPTERUS[1]

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The study of ionizing radiations and their effects on living systems has been of interest to biologists for many years. An important manifestation of such effects is evident in the mitosis of cells or tissues exposed to radiation, and excellent experimental material for the analysis of cytological and developmental consequences of such exposure is available in the eggs of a number of marine invertebrate animals. One of the more favorable forms is the polychaete annelid, *Chaetopterus pergamentaceus*. The eggs of this form, although not transparent, are characterized by moderate amounts of yolk, so that it has been possible to make whole-mount preparations of the irradiated and control eggs which show pertinent cytological detail clearly. Furthermore, the normal development of Chaetopterus has been studied by Mead (1898) and Lillie (1906), and there are available time-tables of development for given temperatures, so that irradiation can be begun at a known stage of mitosis, and subsequent deviations from the normal schedule of events studied in considerable detail.

Materials and Methods

Ripe Chaetopterus males and females were collected by the M. B. L. Supply Department, and maintained in the laboratory in separate large fingerbowls supplied with running sea water. Eggs were obtained by clipping off two or three posterior parapodia from a single female; these parapodia were transferred to pieces of cheesecloth wet with filtered sea water, through which the eggs passed into 100 cc. of filtered sea water contained in each of two (control and experimental) plastic boxes, 3¼″ × 2¾″ × 1¼″ in size and provided with lids. The eggs were allowed to stand undisturbed for 15 minutes; during that period, the first maturation division proceeds to the metaphase (Lillie, 1902). Sperm were obtained by placing one parapodium from a male in 100 cc. of filtered sea water, 15 minutes before they were to be used for insemination.

Immediately after insemination, the lids were placed on both boxes of egg suspension, and exactly thirty minutes after insemination, the experimental eggs were irradiated by the Laboratory x-ray technician, Mr. Alan P. Brockway. Samples from both control and experimental groups were examined at intervals with a binocular dissecting microscope, to ascertain the progress of cleavage and later development. After irradiation, the contents of control and experimental boxes were transferred to two small fingerbowls, each containing an additional 100 cc. of

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filtered sea water; both fingerbowls were then kept on the sea water table, with running sea water flowing around them. The room air temperature varied from 21 to 25° C. Control and experimental cultures were examined for the final time 19 to 22 hours after insemination, when the controls were vigorously swimming trochophores.

The x-ray generator used operates simultaneously two Coolidge tubes at 25 ma and 182 KVP, with an inherent filtration of 0.2 mm. copper. The plastic box (from which the lid was first removed) containing the experimental eggs was thus "cross-fired" between two x-ray beams. Since the eggs were distributed in an even layer on the bottom of the box, a fairly uniform field of irradiation was possible. The x-rays were delivered at two different rates. For total dosages below 3570 r, the machine was calibrated at 510 r per minute with the two tubes 48 cm. apart, the bottom of the Lucite box being approximately equidistant between the two tubes. For dosages above 3570 r, the x-rays were delivered at a rate of 2160 r per minute, with the tubes 16 cm. from one another. There was no appreciable rise in temperature during any of the irradiation treatments, and artificial cooling measures were therefore not used. The duration of treatment varied from one-half minute to eight minutes, and the dosages used were 255 r to 17,280 r

Cytological preparations were made of samples from the control and experimental cultures, at times calculated to result in fixation of the eggs at the metaphase or anaphase of the first three cleavages. A modified squash whole-mount technique was devised, which yielded excellent preparations in a minimum of time. The method involved fixation of the eggs in Kahle's fluid [2] (water, 30 parts; 95% alcohol, 16 parts; formalin, 8 parts; glacial acetic acid, 1 part) on cover-slips. A single drop of concentrated egg suspension was placed on one clean No. 1 square cover-slip, and a drop of fixative on a second cover-slip; the second cover-slip (with the fixative) was inverted and dropped face-to-face onto the first cover-slip. No pressure was applied, and if drops of the correct size were used there was no distortion of the eggs. After about five minutes, the two cover-slips (still face-to-face) were transferred as a unit to a small staining jar containing fresh fixative, where they were kept for 15 to 30 minutes. During this period, the cover-slips were carefully separated from one another with watchmaker's forceps; approximately one-half the eggs adhered to each of the two cover-slips, and very few were lost during the process of separation. After separation, the cover-slips were placed in porcelain racks, Chen Type A, provided with wire handles, and subsequent steps were carried out by transferring the racks to tall Stender dishes containing the necessary reagents. Hydration of the eggs through 70% alcohol, 50% alcohol and distilled water was followed by staining for four to six minutes in a solution of Harris' acid haematoxylin, diluted 1:4 or 1:5 with distilled water and filtered before each use. No counterstain was used. After staining, the eggs were "blued" in several changes of tap water alkalinized with 1% sodium bicarbonate solution, and dehydrated in three changes of triethyl phosphate,[2] in carbol-xylol and in two changes of xylol; they were mounted in damar. The preparations were studied at magnifications of 150 ×, 300 × and 660 ×, using a Spencer compound binocular microscope with compensating oculars. Approximately 440 slides were prepared and studied.

[2] We are indebted to Dr. Anna R. Whiting for suggesting the use of these reagents.

Results

The results are summarized in Table I.

It is apparent that relatively low doses of x-rays (255 to 1020 r) had no immediately obvious effects on fertilized Chaetopterus eggs. There was a very slight retardation (3–5 minutes) of the first three cleavages in the experimental eggs after 765 r, as compared with the control eggs, but no other visible gross or cytological effects. However, when the control and experimental cultures were examined 19 to 22 hours after insemination, the trochophores were very abnormal in cultures which had received 500 and 765 r, respectively. They were, for the most part, very disorganized masses, with marked ciliary defects; they moved feebly, if at all. Almost all the larvae were dead in the groups which had received 1020 r, and disintegration of the undifferentiated cytoplasm had occurred.

In most of the experiments where the eggs were treated with 1275 r and higher dosages, there was at least a slight retardation (5–10 minutes) of the first three cleavages, and the majority of the embryos were dead 19 hours after insemination. The few surviving trochophores were very abnormal, with ciliary defects, cytoplasmic blebs, etc.

The occurrence of cleavage retardation reported here is in accordance with the results obtained by other workers. Thus, Packard (1918) observed delays in the division of Chaetopterus eggs irradiated with gamma rays from radium. Cook (1939) demonstrated a two- to five-hour delay in the first cleavage of Ascaris eggs x-irradiated at the one-cell stage. Henshaw (1940a, 1940b) and Henshaw and Cohen (1940) described marked cleavage retardation in Arbacia eggs, after x-irradiation of eggs, or sperm, or both gametes. Carlson (1938) described a cessation of mitosis in grasshopper neuroblasts treated with varying doses of x-rays (100–1000 r). The recovery time for return of mitosis (anaphases being used as the criterion) varied from three hours after 100 r to 22 hours after 1000 r. There must be a considerable difference in the radio-sensitivity of Chaetopterus eggs, as opposed to grasshopper neuroblasts, since complete inhibition or even pronounced delay of mitosis in our experiments required much higher dosages.

X-ray dosages below 8640 r did not result in any obvious cytological damage to eggs fixed at the times of the first three cleavages; at and above that dose level, however, there were often multipolar spindles, chromosome bridges at anaphase, and fragmentation, particularly in eggs fixed at the time of the third cleavage. These were very similar to the aberrations described by Costello, Henley and Kent (1952) in P^{32}-treated fertilized Chaetopterus eggs. The chromosomes in the Chaetopterus egg are small, and the detection of minute cytological abnormalities is not always possible.

Eggs treated with 15,120 r presented a striking cytological picture when they were fixed at the time of the second and third cleavages. At least some degree of karyokinesis had apparently taken place, but it was not accompanied by cytokinesis, so that there were often several interphase nuclei present in one undivided or incompletely divided mass of cytoplasm. The nuclei were elongated and somewhat pear-shaped, with the narrow "stem" of one of a pair of nuclei directed toward the narrow "stem" of what was apparently its sister nucleus. One had the impression that an abnormal mitotic division with, apparently, a thick chromatin bridge, had been arrested during its course. This phenomenon of nuclear division without

TABLE I—*X-irradiation of fertilized Chaetopterus pergamentaceus eggs*

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X-ray dose*	Gross abnormalities noted at stage of:				Cytological abnormalities noted in eggs fixed at:		
	1st cleavage	2nd cleavage	3rd cleavage	Trochophores	1st cleavage	2nd cleavage	3rd cleavage
255 r	None	None	None	Very slight: a few dead	None	None	None
510 r	None	None	None	Abnormal: ciliary defects, cytoplasmic blebs	None	None	None
765 r	Slight retardation**	Slight retardation	Slight retardation	Abnormal: ciliary defects, cytoplasmic blebs	None	None	None
1020 r	None	None	None	Mostly dead; those living very abnormal	None	None	None
1275 r	Retardation	Retardation	Retardation	Mostly dead; those living very abnormal	None	None	None
1530 r	Slight retardation	Slight retardation	Slight retardation	Dead	None	None	None
2550 r	Slight retardation	Slight retardation	Slight retardation	Mostly dead; those living very abnormal	None	None	None
3570 r	None	Slight retardation	Slight retardation	Dead	None	None	None
8640 r	Retardation	Retardation	Abnormal 2- and 3-cell stages	Dead	None	None	Multipolar spindles; chromosome fragments and bridges
12,960 r	Retardation	Retardation	Retardation	Dead	None	None	Multipolar spindles; chromosome fragments and bridges
15,120 r	Retardation	Retardation	Retardation	Mostly dead; those living very abnormal	None	Nuclear division without division of cytoplasm. Many very elongated interphase nuclei	Multiple interphase nuclei in undivided or partially divided cytoplasm. Many elongated nuclei
17,280 r	Retardation	Inhibition (most eggs not cleaved)	Inhibition (most eggs 2- and 3-cells)	Dead	None	Undivided eggs	Two interphase nuclei present in each of the 2 cells in most cases. Some 3-cell stages with 1 interphase nucleus per cell; AB cell is one which appears to have divided

* X-ray data: Inherent filtration 0.2 mm. Cu; 25 ma; 182 KVP. Two tubes: 48 cm. apart, 510 r per minute, for doses up to 3570 r; 16 cm. apart, 2160 r per minute, for doses above 3570 r; irradiated eggs equidistant between tubes in all cases.

** Cleavage retardation times were ascertained only approximately; in all cases they varied from 4 to 15 minutes, except in those groups in which cleavage was completely inhibited.

cytoplasmic division, or with incomplete cytoplasmic division, is perhaps the most striking effect noted after treatment of these eggs with high dosages of x-rays.

X-irradiation with 17,280 r resulted in marked effects (both gross and cytological) on the first three cleavages. The division to two cells was very abnormal and noticeably retarded (5–7 minutes), and the two subsequent divisions were almost completely inhibited. There were some two- and three-cell stages present in the samples fixed at the times of second and third cleavage in the controls; the stages fixed 75 minutes after insemination often had two interphase nuclei in each of two cells, again indicating a suppression of cytokinesis at an earlier stage. The three-cell stages (which are not normally found in the cleavage of Chaetopterus, although the polar lobe may simulate a third cell) usually had only one interphase nucleus in each of the three cells. It appeared that cytoplasmic division had taken place only in the smaller AB blastomere at the second cleavage, the larger CD blastomere remaining undivided. The possible significance of this observation will be discussed below.

Discussion

Sensitivity of prophase chromosomes to irradiation

The design of our experiments was such that treatment was begun at prophase of the first cleavage, a time which appears, according to the findings of many observers utilizing a variety of materials, to be very susceptible to radiation. This fact, coupled with the circumstance that division in Chaetopterus eggs is predictable and synchronous (within certain limits imposed by temperature and other environmental factors), makes the material favorable for studies of radiation effects.

One of the early reports on the susceptibility of prophase chromatin to irradiation came from Strangeways and Hopwood (1926), who irradiated chick tissue cultures with x-rays and found the most sensitive period to be at the time immediately before the onset of visible prophase. Sax (1938, 1943) reported the greatest frequency of x-ray-induced chromosome aberrations in Tradescantia microspores which were at the meiotic or mitotic prophase at the time of treatment. Luther (1938) x-irradiated frog eggs, and concluded that the maximum susceptibility was during prophase, the minimum susceptibility at metaphase. The findings of Marshak (1939) are somewhat at variance with the general observation that prophase is the stage of greatest sensitivity; x-irradiated onion seedlings, pretreated with dilute solutions of ammonia, were examined cytologically and the results interpreted to indicate that the chromosomes were most sensitive at the resting stage. This was held to be the case regardless of whether or not ammonia pre-treatment was used.

Henshaw and Cohen (1940) x-irradiated fertilized Arbacia eggs, and found that when cleavage retardation was the criterion, the time of greatest susceptibility was during the period after the male and female pronuclei had come together, and during early prophase. The late prophase stage, immediately before breakdown of the nuclear membrane, was found by Carlson (1941) to be the most sensitive period for grasshopper neuroblast cells, and Swanson (1942) made similar observations on Tradescantia pollen tube chromosomes.

More recently, Bloom, Zirkle and Uretz (1955) utilized microbeams of protons and ultraviolet light for irradiation of individual chromosomes and parts of chromo-

somes, in cultures of Triturus heart tissue. They found that a given dose at the prophase stage produced "sticky" chromosomes and chromosome fragmentation; the same radiation to metaphase chromosomes resulted, again, in sticky chromosomes but very few akinetic chromosomes or fragments were observed. Their findings are of great interest, not only because of the highly localized character of the radiation used, but also because they were able to follow the fate of a single irradiated chromosome in considerable and exact detail by the use of phase optics and time-lapse motion pictures.

When Chaetopterus eggs are x-irradiated beginning 30 minutes after insemination, both polar bodies have usually been given off (except in a few of the experiments performed early in the breeding season, when low temperatures slowed down all features of development of the egg). At 21° C., according to Heilbrunn and Wilson (1948), the male and female pronuclei have approached one another and are fusing, and by 40 minutes after insemination, the fusion nucleus is at the prophase of the first cleavage. This time-table of events proceeds at a considerably faster rate when the temperature is increased, and our data indicate that for most of the experiments reported here, the eggs were at the early prophase stage when irradiation was begun.

Possible effects of irradiation on early development of the eggs

In the ovary of the Chaetopterus female, a definite polarization of the egg is evident, the future animal pole being free in the lumen of the ovarian tubule, and the future vegetal pole being attached to the wall of the tubule (Lillie, 1906). Thus, the subsequent position of the polar bodies is "set" very early in development. The cleavage spindle is formed by the separation of the two sperm centrosomes (Mead, 1898), in a position in the egg which is determined by the position of the male pronucleus. This, in turn, is determined by the polarity of the egg (the sperm nearly always entering in the vegetal hemisphere), so that there is an orderly chain of events, each one of which is predicated on the original polarity of the egg.

Lillie (1906) described the separation of large basophilic granules from the chromosomes of the first cleavage in Chaetopterus. The subsequent division of the egg segregates all these granules into the CD blastomere, none being left in the AB cell. X-irradiation with 17,280 r at prophase resulted, as noted above, in a failure of the CD blastomere to divide at the second or at the third cleavage, so that there appears to have been a drastic effect on the distribution of these granules, as well as on other cytoplasmic movements.

Indeed, irradiation of eggs at the prophase of first cleavage could very well have other far-reaching effects, quite apart from those on the chromosomes. The influence of x-irradiation on viscosity changes during mitosis of the Arbacia egg has been described by Wilson (1950). In unirradiated eggs, there is a marked increase in viscosity which reaches a value three or four times that of the unfertilized eggs at 15 minutes after insemination; this increased viscosity persists for a few minutes, and then drops to almost the level of viscosity of unfertilized eggs shortly before the first cleavage. Approximately the same magnitude of increase was observed in eggs irradiated and then inseminated, but it remained high for a period two to three times that of the control eggs, eventually decreasing shortly before

cleavage. If viscosity changes occur in the fertilized Chaetopterus egg irradiated after insemination, they would be expected to have profound effects on the complex patterns of oöplasmic segregation characteristic of this form. Lillie (1906) demonstrated that there was an exact correlation between the distribution of ectoplasmic spherules (which, before the breakdown of the germinal vesicle, are distributed over the animal two-thirds of the egg, and which come to overflow the vegetal hemisphere after germinal vesicle breakdown) and the distribution of cilia in the trochophore larvae. The cilia apparently do not develop directly from the spherules, however. The fact that the later stages of development in our experimental cultures were almost invariably marked by abnormalities of cilia distribution (if, indeed, the embryos survived to the stage of cilia formation at all) indicates that the irradiation directly or indirectly affected some part of the cilia-producing mechanism.

The phenomenon of polar lobe formation, which is characteristic of the eggs of certain annelids (including Chaetopterus) and molluscs, is a striking indication of the profound changes which occur in the cytoplasm of these eggs within the first few hours after fertilization. Although we observed no visible evidence of malformation of the polar lobe in the irradiated eggs, it is quite possible that there were inconspicuous disturbances in the apportionment of cytoplasmic materials to this structure. It is of interest in this connection to point out that even after 17,280 r, polar lobe formation immediately before the first cleavage was morphologically normal, although delayed. Formation of the second polar lobe after this dosage appears to have been completely suppressed.

In any event, the deleterious effects of x-ray treatment on later development are, *per se*, an indication that irradiation interfered with early processes of differentiation.

The possible role of cytoplasmic effects in radiation damage has been considered in the recent paper by Ord and Danielli (1956), in which they transferred x-irradiated *Amoeba proteus* nuclei to non-irradiated cytoplasm of the same form. The resulting organisms had a somewhat lower percentage of survival than did intact irradiated amoebae. When non-irradiated nuclei were transfered to irradiated cytoplasm, 18–48 hours after irradiation, the animals survived despite the fact that they had received very high doses of x-rays; they were able to form new clones, although the time of first division was somewhat delayed. If the transfers of normal nuclei to irradiated cytoplasm were done sooner than 18 hours after treatment, however, the normal nuclei were apparently lethally damaged by the irradiated cytoplasm in most cases. Ord and Danielli point out that in this study, nuclear damage appears to be a *direct* result of x-ray damage to the nuclei, and an *indirect* result of contact with damaged cytoplasm. They suggest that perhaps the importance of nuclear damage from radiation, as opposed to cytoplasmic damage, may have been somewhat over-emphasized.

Chromosome aberrations

There have been many reports in the literature of chromosome aberrations which occur after irradiation from various sources. An early one was the paper by Packard (1918); he irradiated unfertilized Chaetopterus eggs with gamma rays from a radium source and observed multipolar spindles at the first cleavage, after

the treated eggs had been inseminated. Packard interpreted these multipolar spindles as being due to polyspermy, which he felt was facilitated in some way by the irradiation. Alberti and Politzer (1924a, 1924b) described and figured a variety of cytological abnormalities in the corneal epithelium of larval salamanders which had been x-irradiated; among these anomalies were chromatin bridges at anaphase, telophase and interphase, akinetic fragments and chromosomes, accessory nuclei, multiple akinetic chromosomes, and multipolar spindles. Similar effects were described by Strangeways and Hopwood (1926) in x-irradiated chick tissue cultures, and by Sonnenblick (1940) in the embryos obtained after mating x-irradiated male and female Drosophila adults. Laznitzki (1943) also x-irradiated chick tissue cultures, and described a number of cytological abnormalities resulting from treatment; however, this author emphasizes the fact that no multipolar spindles were observed in irradiated material, which is in contrast to the findings of other investigators. Zirkle and Bloom (1953), utilizing proton bombardment of parts of amphibian heart cells in tissue culture, reported chromosome bridges, inhibition of cytokinesis, and unequal distribution of the daughter chromosomes. Chromosome fragmentation after irradiation has been described by Carlson (1938) for grasshopper neuroblast cells, by Fabergé (1940) for Tradescantia pollen grains, by Bishop (1942) for grasshopper spermatocytes, by Whiting and Murphy (1956) for Habrobracon oöcytes, and by Lesley and Lesley (1956) in plants from treated tomato seeds. We have observed many of the aberrations described above in our material, especially after the higher doses of x-rays.

Henshaw (1940d) studied the multipolar spindles which occurred in fertilized Arbacia eggs after x-irradiation; it is interesting that in contrast to our findings (where multipolar spindles clearly attributable to irradiation were observed no sooner than the third cleavage), he described such effects at the first cleavage. This difference may be due to the fact that Henshaw used considerably higher doses (31,200 r) than were employed in the present study. In the same paper (1940d), he found no evidence of nuclear division without accompanying cytoplasmic division at the first cleavage in his treated eggs. We found karyokinesis without cytokinesis only at the second and third cleavages, which apparently were not studied by Henshaw.

Recently, Bloom, Zirkle and Uretz (1955) irradiated parts of chromosomes of amphibian heart cells in tissue culture, using microbeams of protons or ultraviolet. Irradiation of the kinetochore region resulted in "drifting" of the chromosome until anaphase when it was incorporated as a lobe on the daughter nucleus, or became a small accessory nucleus. Chromosome "stickiness" and fragmentation were also reported. Chromosomes treated with beams of protons or ultraviolet outside the kinetochore region showed no such effects. Bombardment of extra-chromosomal areas of the cells (cytoplasm and the ends of spindles) with relatively large numbers of protons produced no effects at the site of irradiation. Heterochromatic ultraviolet irradiation of the same regions, however, resulted in a disappearance of the spindle and derangement of the characteristic metaphase configuration; a "false anaphase" followed, in which chromosomes, rather than chromatids, moved apart.

It is of interest that many of the chromosome aberrations typical of radiation damage are produced also by a variety of other agents including, for example, low temperature (Callan, 1942; Böök, 1945; Henley, 1950; Henley and Costello, 1949);

high temperature (Briggs, 1947); colchicine (Callan, 1942); and ribonuclease (Kaufmann, MacDonald and Bernstein, 1955).

Androgenesis after irradiation

There has been considerable discussion in the literature as to the possibility of androgenesis occurring in fertilized irradiated eggs, as a consequence of irreparable damage to the egg chromatin, so that development proceeds with only the haploid, paternal complement of chromosomes. Packard (1918) reported that in Chaetopterus eggs which had received heavy doses of gamma radiation before insemination, the female pronucleus remained in a polar position, often attached to the polar body material by a fine cytoplasmic strand in which chromatin threads can be distinguished. Cleavage took place in such eggs in an orderly fashion, however, and the haploid number of chromosomes was present. Whiting (1948, 1955) found haploid androgenetic males in Habrobracon. They developed only from eggs x-rayed in first meiotic metaphase, thereby resembling Packard's results in Chaetopterus.

Henshaw (1940d), on the other hand, reported that both pronuclei participated in the development of irradiated Arbacia eggs, even after heavy doses of x-rays. However, Arbacia eggs are fully mature, with the female pronucleus present, when in the fertilizable condition, whereas Chaetopterus eggs are at the metaphase of the first maturation division.

Whiting (1955) studied Feulgen preparations of Chaetopterus eggs irradiated (during metaphase I) with 60,000 r and fertilized 3½ minutes later with untreated sperm. The majority underwent continued cleavage, and of these 26% appeared to have sperm chromosomes, only, and were therefore androgenetic. Whenever chromatin abnormalities appeared in cleaving cells, there were more than nine chromosomes (the haploid number) present, and in all cells in which there were nine chromosomes, only, no aberrations were visible. Whiting therefore found no evidence of an injurious effect of irradiated cytoplasm upon untreated chromosomes. In the experiments reported in the present paper, apposition of the pronuclei occurred normally in irradiated eggs, and the diploid number of chromosomes appeared to be present. Since the eggs had been inseminated before irradiation, the occurrence of normal fertilization implies that no damage was suffered by the sperm, either directly or as a consequence of its passage through irradiated egg cytoplasm.

The effects of irradiation on later development

Lea (1955) points out that the death of a cell as a result of irradiation usually does not occur immediately but at, or following, the next division of the cell; an immediate lethal effect requires much larger doses of radiation than a delayed lethal effect. The results obtained in the present study confirm this general statement, and are in accordance with the findings of other investigators. Cook (1939), for example, x-irradiated Ascaris eggs at the one-cell stage and obtained highly abnormal embryos, consisting of unorganized masses of cells. Sonnenblick (1940) reported the occurrence of undifferentiated non-viable masses of cells among the progency of adult fruit flies which had been treated with x-rays. Henshaw (1940c) found that x-irradiation of Arbacia eggs with 14,400–28,800 r resulted in the

appearance of a wide gradation of effects at the pluteus stage, ranging from shortening of the skeletal arms to the formation of a disorganized mass of cytoplasm which subsequently disintegrated. Giese (1946) treated Chaetopterus sperm with 8000–16,000 ergs/mm² of ultraviolet; when such sperm were used to inseminate normal eggs, death ensued, at a stage which is not specified.

Even after x-ray doses as low as 255 r in our experiments, there was some mortality in Chaetopterus trochophores examined approximately 22 hours after insemination. Above that dose level, the larvae were very abnormal or, more commonly, dead. This delayed action was especially striking after some of the lower doses of x-rays, where no particular gross or cytological effects (except for slight cleavage retardation) were discernible at earlier stages after treatment.

Summary

1. Fertilized eggs of Chaetopterus were x-irradiated, beginning 30 minutes after insemination; doses from 255 r to 17,280 r were used, and the duration of treatment was one-half minute to eight minutes. Observations were made of both living and fixed eggs, at various intervals after irradiation, and of living trochophore larvae 19–22 hours after irradiation.

2. The principal effects of relatively low doses (255–1020 r) were found to be a slight retardation of cleavage (3–5 minutes), and the production of abnormal trochophore larvae which were characterized by severe ciliary defects, cytoplasmic blebs, and very feeble movements (following doses of 255 to 765 r). Doses of 1020 r and above resulted in death of most of the larvae by the trochophore stage. The majority of the eggs irradiated with 1275 r and above showed at least a slight retardation of the first three cleavages.

3. Among the cytological abnormalities observed (especially after the higher doses) were multipolar spindles, chromosome fragmentation, and karyokinesis without cytokinesis.

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THE TAXONOMY OF UNARMORED DINOPHYCEAE OF SHALLOW EMBAYMENTS ON CAPE COD, MASSACHUSETTS [1]

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Several papers give brief accounts of unarmored Dinophyceae found along the eastern coast of the United States. Calkins (1902) described from Woods Hole three European species, with one as a new variety. Herdman (1924a) listed five European, sand-living species from Woods Hole. Lackey (1936) listed thirteen species, all European, in his account of Woods Hole protozoa. Martin (1929) described thirteen species, four of which were new, from Barnegat Bay. It would be expected that further study would show many more extensions of range from the east to the west side of the Atlantic. One wonders, though, whether new species would be few, as suggested by these figures, or on the contrary would be many, since studies hitherto have not been very detailed. Further, the Barnegat Bay list suggests that the shallow, estuarine type of habitat has as many species as coastal waters, since Martin's number is matched only by that of Lackey.

The following study covers twenty-six species, of which twelve are completely

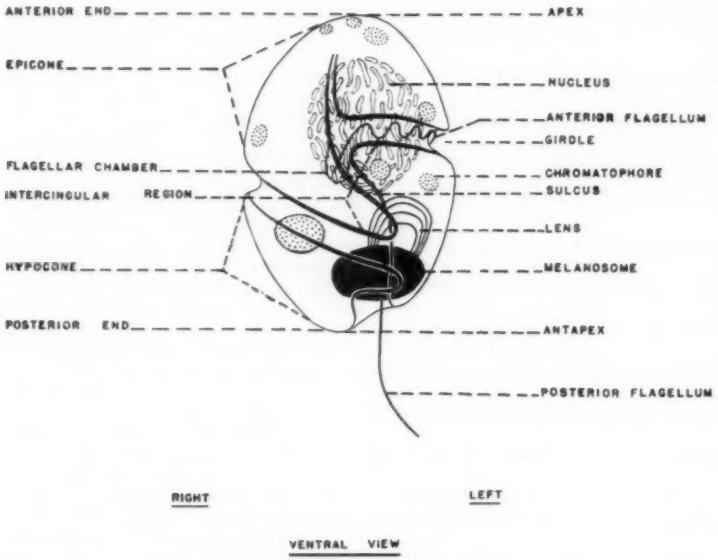

FIGURE 1. Structure of a dinophycean.

[1] Contribution No. 838 from the Woods Hole Oceanographic Institution.

new and nine show extensions of range from Europe. The collections which furnished the great majority of the material were from Great Pond, Falmouth Harbor, Salt Pond, and Uncatena Island Pond, all very shoal embayments on the southern shore of Cape Cod. The expectations, consequently, seem to be fulfilled.

Water samples were concentrated by centrifuging and the Dinophyceae were studied alive in water mounts. At magnifications of × 440 and × 980 drawings were made of specimens which had ceased to swim but showed no deformity due to approaching disintegration. Outline, girdle, and sulcus were drawn with camera lucida; the rest of the structures were drawn free-hand.

For those unacquainted with the morphology of unarmored Dinophyceae and its special nomenclature, Figure 1 illustrates the typical structure of such a dinophycean.

The species studied fall into eight genera, which may be characterized as in the following key:

KEY TO GENERA

Girdle and sulcus rudimentary ..... *Oxyrrhis*
Girdle and sulcus well-developed:
  Solitary:
    Without ocellus and nematocysts:
      Girdle not markedly displaced:
        a) Girdle in anterior third of body ..... *Amphidinium*
        b) Girdle in central portion of body ..... *Gymnodinium*
        c) Girdle in posterior third of body ..... *Massartia*
      Girdle markedly displaced ..... *Gyrodinium*
    With ocellus but without nematocysts ..... *Warnowia*
    With ocellus and nematocysts ..... *Nematodinium*
  Colonial ..... *Polykrikos*

OXYRRHIS Dujardin

Oxyrrhis marina Dujardin

Oxyrrhis marina Dujardin in Kofoid and Swezy, 1921, p. 117, text fig. R, 3.
Oxyrrhis marina Dujardin in Lebour, 1925, p. 19, pl. 1, figs. 6a–6e.

Woods Hole area: Uncatena Island, Salt Pond; March, August, October. New Jersey; White Sea; England; brackish estuary near Nieuport, Belgium; Marseilles harbor; Genoa harbor.

This species is distinguished by the posterior position of its flagella, by the broad excavation of the posterior sulcus, divided midway by a tentacle-like lobe, and by the partial encirclement of the girdle. Other characters are its elongate ellipsoidal form and absence of chromatophores.

AMPHIDINIUM Claparède and Lachmann

KEY TO SPECIES

Chromatophores present:
  Chromatophore single ..... *A. carteri*
  Chromatophores many ..... *A. wislouchi*
Chromatophores absent:
  Body stout, with rounded ends ..... *A. crassum*
  Body very slender, with pointed ends ..... *A. sphenoides*

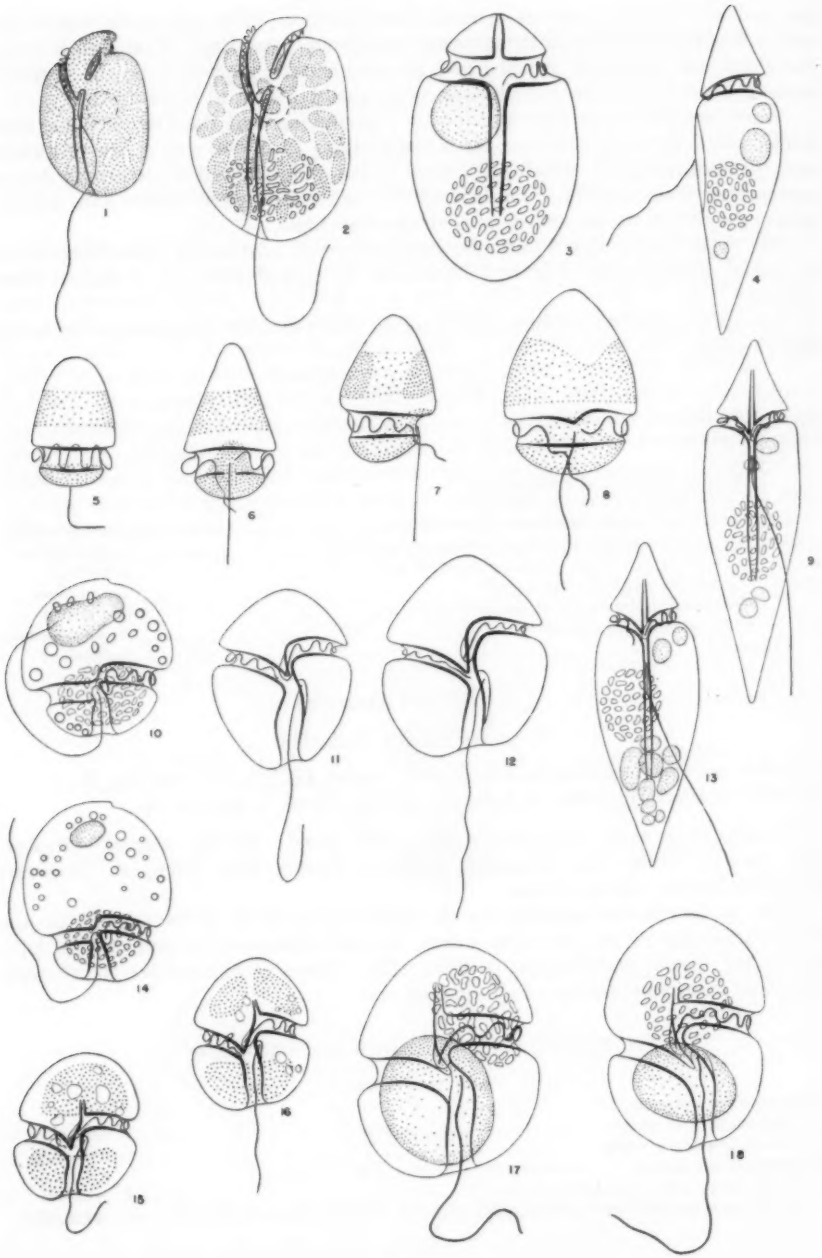

PLATE 1

Amphidinium **carteri** nom. nov.

(Plate 1, Figure 1)

Amphidinium klebsi Carter, 1937, p. 58, pl. 8, figs. 12, 13, 14, 15. (*non* Kofoid & Swezy, 1921).

Body dorsi-ventrally flattened, oval in outline in ventral view, elongate-elliptical in lateral view. Length 12–15 μ, width 8–9 μ. Epicone small, asymmetric, crescent-shaped in ventral view, somewhat flattened at apex; in dorsal view beak-like, rising near right margin and projecting toward left margin. Hypocone truncate-elliptical in ventral or dorsal view, asymmetric, with its right margin convex and left straight or very slightly convex, the antapex broadly rounded. Left limb of girdle starting some distance above posterior end of epicone, running in an arching course anteriorly and laterally, then extending transversely around dorsal surface of epicone, finally leading posteriorly near right margin to round posterior end of epicone, failing, however, to meet the end of the left limb. Sulcus nearer right margin, extending from posterior end of epicone in curving course to antapex. Anterior flagellum inserted at the end of the left limb of girdle; posterior flagellum inserted just below the anterior, separated from it by a "bridge" that separates girdle ends, extending 1.5 body lengths.

Chromatophore single, covering whole inner surface, often perforate, golden brown. Nucleus at posterior end of hypocone, containing short chromatin corpuscles. Pyrenoid present, in center of hypocone. Assimilate granules present or absent.

Woods Hole area: Uncatena Island; October, July. Isle of Wight, England, in brackish pool.

The species described here as *Amphidinium carteri* is identical with Carter's *A. klebsi*. It is considered an independent species since it is much smaller than the forms described as *A. klebsi* by Kofoid and Swezy (1921), Herdman (1924a), and Lebour (1925), and since it has only a single chromatophore.

Amphidinium **wislouchi** n. sp.

(Plate 1, Figure 2)

Amphidinium sp. Wislouch, 1924, p. 121, pl. 3, fig. 11.

Body dorsi-ventrally flattened, oval in outline in ventral view. Length 20–25 μ, width 14–16.5 μ. Epicone small, asymmetric, crescent-shaped in ventral view,

PLATE 1

FIGURE 1. *Amphidinium carteri* nom. nov.
FIGURE 2. *Amphidinium wislouchi* n. sp.
FIGURE 3. *Amphidinium crassum* Lohmann
FIGURES 4, 9, 13. *Amphidinium sphenoides* Wulff
FIGURES 5, 6, 7, 8. *Massartia rotundata* (Lohmann) Schiller
FIGURES 10, 14. *Massartia asymmetrica* (Massart) Schiller
FIGURES 11, 12. *Gyrodinium metum* n. sp.
FIGURES 15, 16. *Gyrodinium estuariale* n. sp.
FIGURES 17, 18. *Gyrodinium glaebum* n. sp.

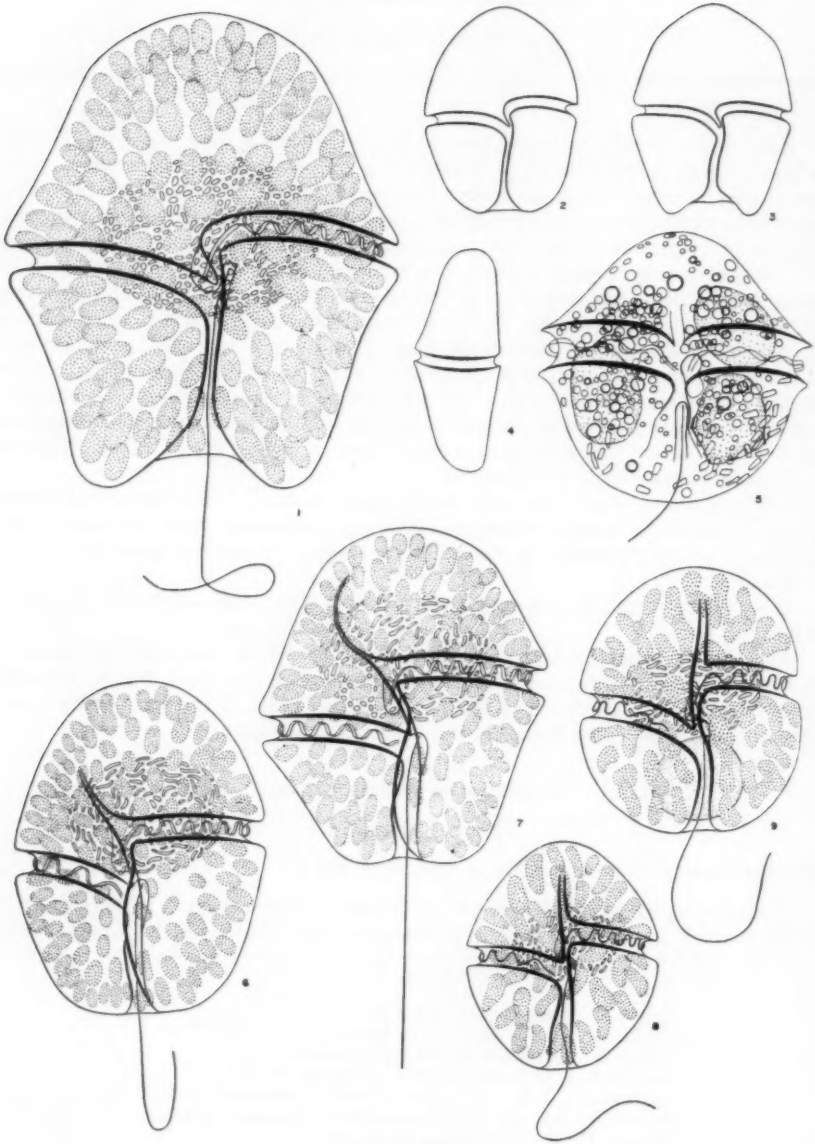

PLATE 2

somewhat flattened at apex; in dorsal view beak-like, rising near right margin and projecting toward left margin. Hypocone truncate-elliptical in ventral or dorsal view, asymmetric, with its right margin convex and left almost straight, the antapex broadly rounded. Left limb of girdle starting some distance above posterior end of epicone, running in an arching course anteriorly and laterally, then extending transversely around dorsal surface of epicone, finally leading posteriorly near right margin to round posterior end of epicone, failing, however, to meet the end of the left limb. Sulcus nearer right margin, extending from posterior end of epicone in curving course to antapex. Anterior flagellum inserted at the end of the left limb of girdle; posterior flagellum inserted just below the anterior, separated from it by a "bridge" that separates girdle ends, extending 1.5 body lengths.

Chromatophores many, elliptical, often arranged in a somewhat radiating manner, with pyrenoid as center.

Woods Hole area: Uncatena Island, Great Pond; October, March. Poland.

This species is about the same as Wislouch's *Amphidinium* sp. and is quite similar to *A. carteri* except for having many chromatophores.

Amphidinium crassum Lohmann

(Plate 1, Figure 3)

Amphidinium crassum Lohmann, 1908, p. 261, pl. 17, fig. 16.
Amphidinium crassum Lohmann in Lebour, 1917, p. 188, fig. 2; 1925, p. 31, pl. 3, figs. 2a–2c.

Body elongate elliptical, circular in cross-section. Length 23–30 μ, width 11–17 μ. Epicone very small, 0.20–0.25 the body length, broadly conical with a pointed apex; hypocone with its sides parallel in the anterior half, rounding into a broad antapex. Girdle not displaced, wide, shallow, its posterior margin wider than the anterior. Sulcus very narrow and shallow, straight, reaching from the apex to 0.66 of the length of hypocone. Flagellar chambers not seen. Anterior flagellum completely encircling body, posterior flagellum not seen.

Chromatophores absent. Nucleus close to antapex, spherical, containing short chromatin corpuscles. Brown ingested bodies and assimilate bodies common.

Woods Hole area: Great Pond, Falmouth Harbor; April, May. Baltic Sea off Kiel; English Channel; Plymouth Sound; Adriatic Sea.

Lohmann (1908) described two similar amphidiniums, *A. crassum* and *A. longum*, the second more slender and with a smaller, more pointed epicone than the first. Lebour (1917, 1925) described as *A. crassum* a type intermediate between these two in proportion of length to width, but with the "fuller" epicone of Lohmann's *A. crassum*. Lebour's type is identical with that described here.

PLATE 2

FIGURES 1, 2, 3, 4. *Gymnodinium nelsoni* Martin
FIGURE 5. *Gymnodinium lazulum* n. sp.
FIGURES 6, 7. *Gyrodinium resplendens* n. sp.
FIGURES 8, 9. *Gyrodinium aureolum* n. sp.

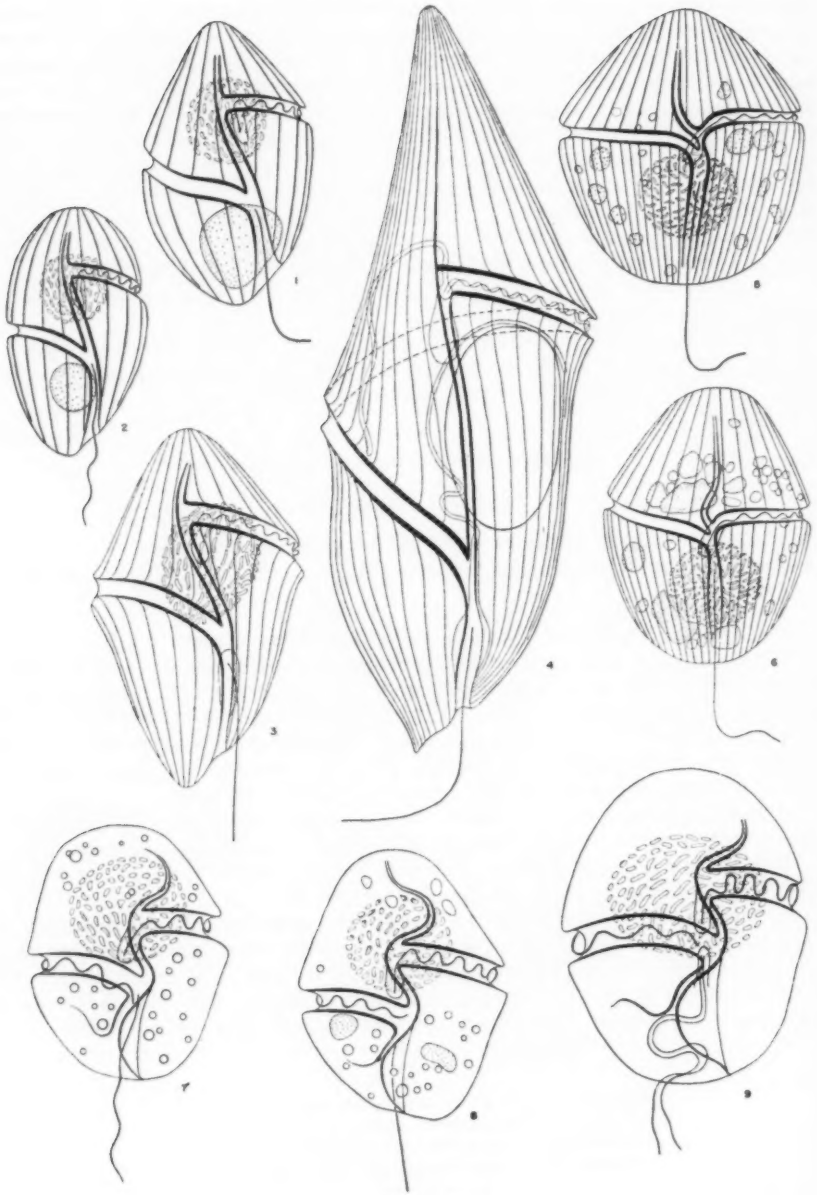

PLATE 3

Amphidinium sphenoides Wulff

(Plate 1, Figures 4, 9, 13)

Amphidinium sphenoides Wulff, 1916, p. 105, pl. 1, figs. 9a–9b.

Body cylindrical, of slender proportions, tapered posteriorly to a sharp point, tapered anteriorly more abruptly to an equally sharp point. Length 35–42 μ, width 12–13 μ. Epicone 0.20–0.25 the total length, symmetrically diamond-shaped or triangular, respectively, in ventral or dorsal view, but asymmetrically triangular in side view, with the dorsal margin more sloping than the ventral; outline contours concave. Hypocone similar but more elongate, triangular in dorsal and ventral view and more sloping along the dorsal than the ventral margin. Girdle deep, broad dorsally but narrowed ventrally, so that in side view the margins appear to converge. Sulcus narrow, running from near the apex to 0.66 the length of the hypocone. Anterior flagellar chamber a mere deepening of the left girdle end (posterior chamber not seen). Anterior flagellum encircling body; posterior flagellum somewhat shorter than the body.

No chromatophores. Nucleus midway the length of hypocone. Assimilate spherules of various sizes present or absent.

Woods Hole area: Great Pond; January. Barents Sea.

GYMNODINIUM Stein (emended by Kofoid and Swezy)

KEY TO SPECIES

Without striations:
- Chromatophores present ..... *G. nelsoni*
- Chromatophores absent:
  - Body globular ..... *G. lazulum*
  - Body laterally flattened ..... *G. stellatum*

With striations ..... *G. striatissimum*

Gymnodinium nelsoni Martin

(Plate 2, Figures 1, 2, 3, 4)

Gymnodinium nelsoni Martin, 1929, p. 14, pl. 3, figs. 25–26.

Body broadly fusoid, with truncate antapex, very much flattened dorsi-ventrally. Length 50–70 μ, width 38–53 μ. Epicone in ventral view sub-hemispherical to somewhat angled, its sides then straight or concave, and its apex broadly pointed. Hypocone trapezoidal, its sides convex, straight, or concave; its apex wide, emarginate to broadly indented. In lateral view dorsal contour somewhat convex, ventral contour somewhat concave; end-on view with a similar dorsal convexity and ventral concavity. Girdle narrow, deep, displaced one-two girdle widths. Sulcus not present on epicone, narrow and sigmoid in intercingular region, straight and

PLATE 3

FIGURES 1, 2, 3. *Gyrodinium dominans* n. sp.
FIGURE 4. *Gyrodinium spirale* (Bergh) Kofoid and Swezy
FIGURES 5, 6. *Gymnodinium striatissimum* n. sp.
FIGURES 7, 8, 9. *Gyrodinium undulans* n. sp.

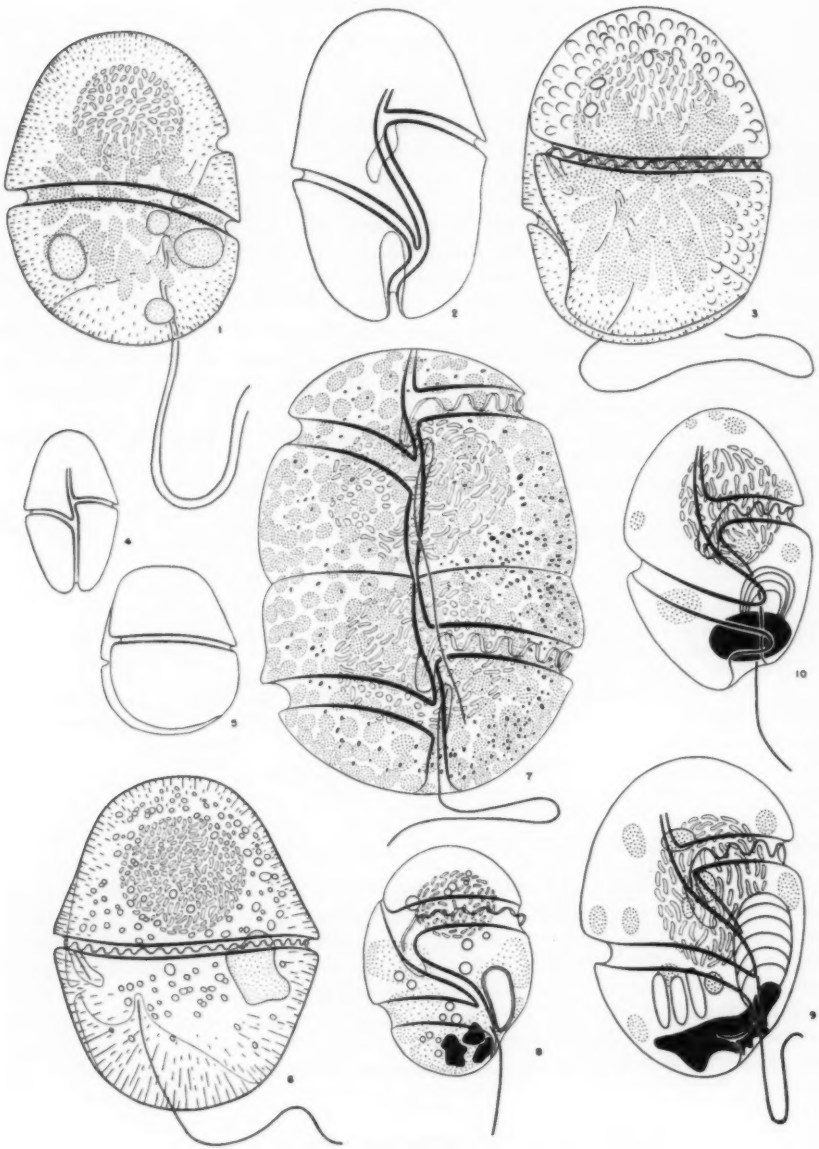

PLATE 4

somewhat wider on hypocone, widening abruptly and showing a large excavation at antapex. Anterior and posterior flagellar chambers overlapping. Anterior flagellum not completely encircling cell; posterior flagellum equal to the body length.

Chromatophores many, rich brown, elliptical, radiating from center of cell. Nucleus central, wider than long, with numberless, elongate chromatin corpuscles. Cells usually free from assimilate and ingested bodies.

Woods Hole area: Great Pond; September. Barnegat Bay, United States east coast.

*G. **nelsoni*** is distinguished from its close relative, *G. splendens* (Lebour, 1925), by having short, elliptical chromatophores instead of elongate slender ones.

Gymnodinium **lazulum** n. sp.

(Plate 2, Figure 5)

Body globular, circular in cross-section, epicone and hypocone subequal. Length 28–30 μ, width 30–32 μ. Epicone conical bell-shaped, with marked concavity of outline near girdle; hypocone hemispherical bell-shaped, with concavity of contours less extensive but closer to girdle than in epicone. Girdle wide, deep, without displacement. Sulcus short, narrow, and faintly defined on epicone; on hypocone abruptly widening, more so on the right than the left, its margins fading out about halfway to antapex. Girdle ends dipping in markedly to the bottom of the sulcus, deeply excavating sulcus in girdle region. Anterior flagellum inserted at opening of a pore; pore extending as small pustule from left girdle end posteriorly. Posterior flagellum inserted in trough-like depression of posterior sulcal floor. Anterior flagellum a delicate band completely encircling body; posterior flagellum short, 0.5 body length.

Chromatophores absent. Nucleus not visible. Cytoplasm clear, quite transparent, smoke blue in color. Colored spherules, grading from copper-red through orange and brown to lemon-yellow, abundant, scattered throughout cytoplasm, often clustered near girdle. Angular, crystal-like, slate-gray bodies scattered at random through hypocone. Large, brown ingested bodies common.

Woods Hole area: Great Pond; December, May.

This species was the only one studied with a clear, blue, instead of a granular, gray, cytoplasm. The array of hues of the numberless small spherules, as well as the blue of the cytoplasm, are matched by similar structures in *G. violescens* (Kofoid and Swezy, 1921).

Gymnodinium **stellatum** n. sp.

(Plate 4, Figures 4, 5, 6)

Body laterally flattened, elliptical in side view with sub-truncate anterior end. Length 25–47 μ, thickness 22–39 μ, width 17–25 μ. Epicone in dorsi-ventral view taller than wide, with slightly sloping sides; in lateral view as wide as tall, trape-

PLATE 4

FIGURES 1, 2, 3. *Gyrodinium uncatenum* n. sp.
FIGURES 4, 5, 6. *Gymnodinium stellatum* n. sp.
FIGURE 7. *Polykrikos hartmanni* Zimmermann
FIGURE 8. *Warnowia parva* (Lohmann) Lindemann
FIGURES 9, 10. *Nematodinium armatum* (Dogiel) Lebour

zoidal, more sloping dorsally than ventrally. Hypocone similar to epicone in shape in dorsi-ventral view, with antapical notch dorsally; in lateral view sub-hemispherical, wider than tall. Girdle narrow, displaced 0.16–0.20 body length. Sulcus narrow, straight except for slight leftward divergence in girdle region, forming a deep hypoconal excavation with anterior limit marked laterally by a vague line running diagonally from posterior dorsal corner toward girdle region on ventral surface. Anterior flagellar chamber a long finger-like pocket extending posteriorly and somewhat dorsally; posterior flagellar chamber a narrow prolongation of hypoconal excavation, reaching almost to anterior chamber. Anterior flagellum encircling less than half the circumference, wide, band-shaped; posterior flagellum body length, sometimes double.

Chromatophores absent. Nucleus within epicone, containing elongate chromatin corpuscles. Peripheral cytoplasm with distinct radial structure. Assimilate occasionally abundant.

Woods Hole area: Salt Pond; October, December, January.

This species is very much like *Gyrodinium uncatenum* in this paper, differing principally in lacking chromatophores, in having a less displaced girdle, and a straighter sulcus. Among hitherto described species, it is similar only to *Gymnodinium bifurcatum* (Kofoid and Swezy, 1921) in its lateral flattening and deep hypoconal excavation.

Gymnodinium striatissimum n. sp.

(Plate 3, Figures 5, 6)

Body globular to elliptical, not flattened, its hypocone slightly larger than epicone. Length 29–43 μ, width 23–31 μ. Epicone conical, pointed, or somewhat truncate. Hypocone similar but more truncate, varying from tapered type with moderately sloping sides and rounded antapex to type with slightly sloping sides and broad antapex. Surface with striations, fewer on epicone (15) than on hypocone (25). Girdle narrow, rather deep, slightly displaced, with distinctive posterior flexure to end of left limb. Sulcus shallow, narrow on epicone, somewhat wider on hypocone, from near apex almost to antapex, distinguished by sharp leftward bend just anterior to girdle. Anterior flagellar chamber prolonged posteriorly. Anterior flagellum completely encircling body; posterior flagellum one body length, sometimes double.

No chromatophores. Nucleus posterior, wholly within hypocone, with elongate chromatin corpuscles. Assimilate bodies of various sizes often abundant.

Woods Hole area: Great Pond; May.

Gymnodinium striatissimum is similar in different number of striations on epicone and hypocone to the much larger *G. multistriatum*, *G. rubrum*, and *G. translucens* (Kofoid and Swezy, 1921).

MASSARTIA Conrad

Key to Species

Without striations:
- Chromatophores present ..... *M. rotundata*
- Chromatophores absent ..... *M. asymmetrica*

With striations ..... *M. glauca*

Massartia rotundata (Lohmann) Schiller

(Plate 1, Figures 5, 6, 7, 8)

Amphidinium rotundatum Lohmann, 1908, p. 261, pl. 17, fig. 9.
Amphidinium rotundatum Lohmann in Wulff, 1916, p. 103, pl. 2, fig. 11.
Amphidinium rotundatum Lohmann in Van Goor, 1925, p. 285, fig. 4.
Gymnodinium minutum Lebour, 1925, p. 45, pl. 5, fig. 4.
Massartia rotundata (Lohmann) Schiller in Conrad, 1939, p. 11, figs. 17–22.

Body top-shaped, circular in cross-section. Length 8–17 μ, width 6–12 μ. Epicone 2.5 times as long as hypocone, conical, with straight to gently convex sides, its apex pointed or somewhat rounded. Hypocone half as long as wide, broadly rounded, in lateral view asymmetric so that the dorsal portion is larger than the ventral one. Girdle very wide, very slightly displaced, its anterior margin overhanging and of greater diameter than the posterior margin. Sulcus not discernible. Flagellar chambers absent. Anterior flagellum up to twice the girdle circumference in length; posterior flagellum equal in length to the cell body.

Chromatophores two, yellow-brown; one band-shaped, partially encircling periphery of epicone; the other filling bottom of hypocone, extending on ventral surface to epicone. Nucleus not seen. Assimilate bodies present or absent. Pellicle occasionally present.

Woods Hole area: Great Pond, Falmouth Harbor; January to April, August, September. Barents Sea; White Sea; Baltic off Kiel; brackish estuaries near Nieuport (Belgium) and along the coast of Holland; Plymouth Sound; Adriatic Sea.

Several different populations were studied. One had straight sides to the epicone and a pointed apex. Another had convex sides to the epicone and a pointed apex. A third was rather variable, rotund in appearance, often almost colorless, with apex rounded or pointed. In a similar way this distinctive species shows considerable variation as it receives treatment from various investigators. Lohmann describes it with relatively very small hypocone, whereas Wulff describes it with relatively very much larger one. In contrast to these, which have pointed apices, Conrad's has a rounded apex. Van Goor shows a very slender form. Lohmann's and Wulff's figures show a lobed epiconal chromatophore, whereas Lebour's shows a band-shaped chromatophore.

Massartia asymmetrica (Massart) Schiller

(Plate 1, Figures 10, 14)

Gymnodinium asymmetricum Massart, 1920, p. 132, figs. 22A–D.
Massartia asymmetrica (Massart) Schiller in Carter, 1937, p. 59, pl. 8, figs. 17–18.

Body globular, oval in outline, compressed dorsi-ventrally. Length 14–22 μ, width 13–20 μ. Epicone 0.66 body length, hemispherical, with small apical notch. Hypocone small, 0.33 body length, twice as wide as long, broadly rounded, often with slight oblique flattening in antapical region. Girdle very wide, shallow, displaced one girdle width, its anterior margin wider than posterior. Sulcus extending from girdle to antapex, widening abruptly during its course. Anterior

flagellum incompletely encircling body; posterior flagellum 1.0–1.5 body lengths.

Chromatophores absent. Nucleus almost wholly within hypocone, wider than long, containing relatively few, short, unoriented chromatin corpuscles. Large, brown ingested body and smaller assimilate bodies frequent.

Woods Hole area: Great Pond; October, January, February. Isle of Wight, England; estuary near Nieuport, Belgium.

Massartia asymmetrica is similar to *M. vorticella* (Stein) Schiller and *M. stigmaticum* (Lindemann) Schiller, which, however, contain stigmas, and to *M. glandula* Herdman, which differs, however, in its larger size (20–35 μ long) and helmet-shaped instead of hemispherical epicone. Massart's and Carter's figures of *M. asymmetrica* show somewhat greater displacement of girdle than in the specimens described here.

Massartia glauca (Lebour) Schiller

Spirodinium glaucum Lebour, 1917, p. 196, fig. 13.
Gyrodinium glaucum (Lebour) Kofoid and Swezy, 1921, p. 308, pl. 9, fig. 94, text fig. DD, 16.
Gyrodinium glaucum (Lebour) Kofoid and Swezy in Lebour, 1925, p. 54, pl. 7, fig. 4, text fig. 15.

Woods Hole area: Great Pond; October, May. Plymouth Sound; Adriatic Sea; La Jolla, California.

The specimens studied agreed closely in shape with those of Lebour and were not like Kofoid and Swezy's somewhat different form. They were, however, considerably smaller than Lebour's, 28–32 μ instead of 40–56 μ in length. This form is different from other species of *Massartia* in its striations; it is distinguished by its slender form, the slight twist to the apex, and its absence of chromatophores.

GYRODINIUM Kofoid and Swezy

Key to Species

Without striations:
- Chromatophores present:
  - Chromatophores 2 to 4 ..... *G. estuariale*
  - Chromatophores many:
    - Body dorsi-ventrally flattened:
      - Sulcus not deflected on epicone ..... *G. aureolum*
      - Sulcus deflected to right on epicone ..... *G. resplendens*
    - Body laterally flattened ..... *G. uncatenum*
- Chromatophores absent:
  - Sulcus sigmoid:
    - Epicone rounded-conical ..... *G. metum*
    - Epicone hemispherical ..... *G. glaebum*
  - Sulcus bisigmoid ..... *G. undulans*

With striations:
- Length 18–43 μ ..... *G. dominans*
- Length 66–96 μ ..... *G. spirale*

Gyrodinium **estuariale** n. sp.

(Plate 1, Figures 15, 16)

Body ellipsoid; apex often somewhat pointed compared to broadly rounded antapex; often with slight asymmetry, the right side more convex than the left; slightly flattened dorsi-ventrally; with equal epicone and hypocone. Length 11–16 μ, width 9–12 μ. Epicone broadly conical to sub-hemispherical, the right margin often a bit more sloping than the left; hypocone hemispherical to somewhat trapezoidal, with rounded to flattened, oblique, sometimes indented antapex. Girdle deep, moderately wide, displaced 0.25–0.33 body length, strongly posteriorly bending in right limb. Sulcus slight on epicone, markedly deflected to right in intercingular area, widening, and proceeding straight to antapex on hypocone. Anterior flagellar chamber an elongate pocket diverging to right and running beneath posterior flagellar chamber which is a leftward underhollowing of sulcus between girdle ends. Anterior flagellum encircling body completely; posterior flagellum body length.

Chromatophores yellow-brown, one or two in epicone, one or two in hypocone, distinctively inset from periphery. Nucleus not seen. Assimilate bodies usually sparse.

Woods Hole area: Great Pond, Salt Pond, Uncatena Island; July, August, October to January.

Gyrodinium estuariale is very similar to *Gymnodinium vitiligo* and *Gymnodinium veneficum* (Ballantine, 1956), differing in greater displacement of girdle ends, in wider, deeper girdle and sulcus, and in an oblique, instead of symmetrically rounded, antapex. In the intercingular region the sulcus is deflected to the right (passing from anterior to posterior end) in these species, contrary to most gyrodinia. *Gyrodinium estuariale* is similar to *Gymnodinium marylandicum* (Thompson, 1947); but the latter's sulcus follows the longitudinal axis or is deflected slightly to the left.

Gyrodinium **aureolum** n. sp.

(Plate 2, Figures 8, 9)

Body essentially globular, its dorsi-ventral outline either somewhat ellipsoidal or somewhat fusiform, slightly dorsi-ventrally flattened, with subequal epicone and hypocone. Length 27–34 μ, width 17–32 μ. Epicone hemispherical to broadly conical, sometimes slightly truncate. Hypocone similar, but usually distinctly truncate, with antapex faintly indented at times. Girdle wide, moderately deep, displaced 0.20 body length. Sulcus reaching from just behind apex all the way to antapex, with slight, left deflection in girdle region, rather narrow on epicone, wide on hypocone. Anterior flagellar chamber a posteriorly pointed, finger-shaped cavity; posterior flagellar chamber an underhollowing of left sulcal margin opposite right girdle limb. Anterior flagellum completely encircling body; posterior flagellum very long, up to two body lengths.

Numerous yellow-brown chromatophores present, elliptical in shape, usually arranged in a somewhat radiating manner. Nucleus spherical or wider than long, with elongate chromatin corpuscles.

Woods Hole area: Great Pond, Uncatena Island, Falmouth Harbor; December to April.

This species is rather similar to *Gyrodinium aureum* (Conrad, 1926). It is, however, different in its less elongate chromatophores, wider grooves, greater width for the same length, more conical outline of epicone and hypocone, and somewhat less displacement of girdle ends.

Gyrodinium **resplendens** n. sp.

(Plate 2, Figures 6, 7)

Body broadly fusoid, with truncate apex and antapex, moderately flattened dorsi-ventrally. Length 36–62 μ, width 32–48 μ. Epicone and hypocone similar, equal, trapezoidal in outline, their sides convex, straight, or concave; the apex somewhat rounded, the antapex with sulcal indentation. Girdle deep, moderately wide, displaced 0.20–0.25 body length. Sulcus extending onto epicone as very narrow superficial groove, diverging to right; in intercingular region narrow, vertical or left deflected; on hypocone running straight to antapex, superficially narrow but broad beneath projecting lappet of left margin. Anterior and posterior flagellar chambers elongate pockets projecting toward but not reaching each other. Anterior flagellum completely encircling body; posterior flagellum one body length.

Chromatophores oval, rich brown, radiating, many-tiered. Nucleus somewhat anterior of center, wider than long, with numerous, elongate chromatin corpuscles. Ingested bodies occasional; assimilate absent.

Woods Hole area: Great Pond; July, August.

This species is close to *Gyrodinium aureolum*. It is also quite like *Gymnodinium nelsoni*, differing in greater girdle displacement (so that it falls into the genus *Gyrodinium*), less dorsi-ventral flattening with no ventral concavity, and truncate rather than hemispherical epicone.

Gyrodinium **uncatenum** n. sp.

(Plate 4, Figures 1, 2, 3)

Body laterally flattened, elliptical to quadrangular in side view, elongate elliptical in ventral or dorsal view, with epicone and hypocone subequal. Length 40–54 μ, width 28–33 μ. Epicone in ventral and dorsal views helmet-shaped, taller than wide, broadly rounded at apex, its sides sloping gently, with slight concavities at girdle; in lateral view, sub-hemispherical to trapezoidal, wider than tall. Hypocone very similar but often slightly more truncate at antapex and more distinctly trapezoidal in side view. Girdle narrow, deep, displaced 0.33 body length, the right limb bending steeply posteriorly. Sulcus projecting slightly on epicone, curving to left in intercingular area, then sharply to right between right girdle end and antapex; carried across antapex all the way to dorsal side; deeply excavating hypocone, the anterior extent of excavation seen laterally as an oblique line running from dorsal end of sulcus toward ventral surface. Anterior flagellar chamber long, finger-like, projecting posteriorly and somewhat dorsally and rightward; posterior flagellar chamber a long extension of sulcal excavation, reaching nearly to anterior chamber. Anterior flagellum wide and strap-shaped, completely en-

circling body; posterior flagellum occasionally double, twice the body length; both flagella reaching all the way to the end of the chambers.

Chromatophores elongate, yellow-brown, radiating from center, leaving marginal area clear (lateral view). Nucleus spherical, in the epicone, with slightly elongate chromatin corpuscles. Crytoplasm showing vague radiating structure in peripheral region. Countless, dark assimilate bodies often present.

Woods Hole area: Great Pond, Uncatena Island; July, August, October.

This species is rendered distinctive by its lateral flattening, a rarity among gyrodinia. Very striking is the deep excavation in the hypocone, identical to that in *Gymnodinium stellatum* and *G. bifurcatum* (Kofoid and Swezy, 1921).

Gyrodinium metum n. sp.

(Plate 1, Figures 11, 12)

Body somewhat asymmetric, the right side more convex than the left, circular in cross-section, with hypocone slightly larger than epicone. Length 14.5–22 μ, width 11–16 μ. Epicone rounded-conical in outline; hypocone truncate with straight to convex sides. Girdle deeply excavated, displaced about 0.20–0.25 body length, its right limb curving steeply posteriorly. Sulcus sigmoid, not extending onto epicone, narrow in intercingular region, and wide and deep on hypocone, flattening or indenting the antapex. Anterior flagellar chamber produced inward and posteriorly; posterior flageller chamber a mere excavation in the sulcal floor. Anterior flagellum completely encircling the body; posterior flagellum 1.5 times body length.

Chromatophores absent. Cytoplasm gray, foamy in texture. Nucleus not seen.

Woods Hole area: Great Pond; May, June, July, December, February.

This species is distinguished by the Chinaman-hat shape of the epicone. A smaller-size variant was often seen. Its features are identical except respecting size—9.5–12 $\mu \times$ 9–7 μ.

Gyrodinium glaebum n. sp.

(Plate 1, Figures 17, 18)

Body elliptical, very slightly compressed dorsi-ventrally, with equal epicone and hypocone. Length 17–25 μ, width 12–19 μ. Epicone hemispherical but slightly asymmetric, with right contour more sloping or less fully curved than left. Hypocone similar, but broader, the asymmetry more marked, the left contour more sloping or less fully curved than right, often with oblique flattening in region of sulcus end. Girdle wide, rather deep, displaced two-three times its own width. Sulcus extending slightly onto epicone, narrow and leftward diverging in intercingular region, wide and deep in a straight course on hypocone. The two flagellar chambers overlapping each other, the anterior one a pronounced, posteriorly directed excavation, the posterior one an underhollowing of left girdle margin. Anterior flagellum only partially encircling body, posterior flagellum as long as body.

Chromatophores absent. Nucleus somewhat anterior of girdle, with large and relatively few chromatin corpuscles. Large, brown ingested bodies often present, as well as small, refractive assimilate bodies.

Woods Hole area: Great Pond; July, October.

Gymnodinium variabile (Herdman, 1924a) is close in shape but lacks any marked girdle displacement.

Gyrodinium undulans n. sp.

(Plate 3, Figures 7, 8, 9)

Body elliptical, somewhat dorsi-ventrally flattened with equal epicone and hypocone. Length 27–38 μ, width 21–31 μ. Epicone varying from sub-hemispherical to truncate-pyramidal, with rounded apex and straight but sloping sides. Hypocone asymmetric, truncate-pyramidal with rounded to flattened, oblique antapex, and sloping sides. Girdle wide, deep, displaced 0.20 body length, its right limb curving strongly posteriorly. Sulcus distinctive, forming a bi-sigmoid curve; very narrow on epicone, swerving leftward and then rightward to meet left girdle end, curving again to left in intercingular area and widening, then bending to right on hypocone to form large overlapping lobe, finally returning leftward to antapex. Floor of sulcus on hypocone extending straight to antapex; and right margin of sulcus "bearing away" laterally to form a swelling. Anterior and posterior flagellar chambers projecting toward, but not reaching, each other. Anterior flagellum completely encircling body; posterior flagellum one body length, occasionally double.

Chromatophores absent. Nucleus large, principally within epicone, of elongate chromatin corpuscles. Assimilate bodies sometimes in form of large blocks.

Woods Hole area: Great Pond; February, January.

Few Dinophyceae have either bi-sigmoid sulci or overlapping sulcal lobes.

Gyrodinium dominans n. sp.

(Plate 3, Figures 1, 2, 3)

Body broadly fusiform, circular in cross-section, epicone and hypocone subequal. Length 18.5–43 μ, width 10–22 μ. Epicone and hypocone conical to rotund-conical, their sides varying from convex to straight. Both epicone and hypocone occasionally with slight concavities near girdle. Surface with continuous striations, the number the same on epicone and hypocone, between 7 and 10 across ventral face. Girdle of moderate depth and width, displaced 0.25–0.33 body length. Sulcus sigmoid, deflected to left 0.25 transdiameter in intercingular region, reaching halfway up epicone, extending to posterior margin of hypocone on left side of antapex. Anterior flagellar chamber a posteriorly directed, finger-like projection from left end of girdle. Posterior flagellum inserted at posterior end of girdle (flagellar chamber not seen). Anterior flagellum not completely encircling body; posterior flagellum short, 0.50 body length.

Chromatophores absent. Nucleus anterior, in epicone, containing elongate, oriented chromatin corpuscles.

Woods Hole area: Great Pond, Falmouth Harbor, Salt Pond; April, July, August, October to December.

Gyrodinium dominans is an ally to three very similar species of *Gyrodinium*: *G. pingue* (Schütt, 1895, as *Gymnodinium spirale* var. *pinguis;* Wulff, 1916, as

Spirodinium varians; Kofoid and Swezy, 1921; Lebour, 1925), *G. obtusum* (Schütt, 1895, as *Gymnodinium spirale* var. *obtusa;* Kofoid and Swezy, 1921; Lebour, 1925), and *G. fissum* (Kofoid and Swezy, 1921). The chief distinction is the flexure of the sulcus in *G. dominans,* contrasting with the comparative straightness of the sulcus in the other three.

Gyrodinium spirale (Bergh) Kofoid and Swezy

(Plate 3, Figure 4)

Gyrodinium spirale (Bergh) Kofoid and Swezy, 1921, p. 332, pl. 4, fig. 43, text fig. DD, 14.
Gyrodinium spirale (Bergh) Kofoid and Swezy in Lebour, 1925, p. 56, pl. 8, fig. 1.

Body slender fusiform; circular in cross-section; somewhat twisted on its longitudinal axis; with epicone longer but narrower than hypocone. Length 66–96 μ, width 30–38 μ. Epicone narrowly conical, convex or straight along left and dorsal contours. Hypocone with sides parallel anteriorly, convex posteriorly; its antapex pointed, excentric, to right of ventral, longitudinal axis; with sulcal notch to left of antapex. Surface with continuous striations, 8–13 on epicone, 15–20 on hypocone. Girdle narrow, rather shallow, displaced more than 0.33 the body length, its right limb curving strongly posteriorly. Sulcus very narrow, from somewhat behind apex to left girdle end as a heavy line, a very narrow but distinct groove between girdle ends, widening and deepening from right girdle end to antapical notch. Anterior flagellum inserted at opening of a pustule, which is composed of a short, sac-shaped portion, extending posteriorly, and of a long, thread-like portion, extending somewhat anteriorly, then laterally, finally posteriorly along right contour to region of right girdle limb. Posterior flagellum likewise inserted at opening of a pustule having a long, thread-like extension anteriorly and leftward to region of left girdle limb. Anterior flagellum following girdle 0.33 or less of girdle length. Posterior flagellum short, about 0.25 of body length.

Chromatophores absent. Nucleus elongate-ellipsoidal on left side (ventral view), in intercingular area, without apparent structure.

Woods Hole area: Great Pond, Falmouth Harbor; December, April, May, August. Baltic Sea; Norway; Port Erin, Ireland; Plymouth Sound; Adriatic Sea; La Jolla, California; Indian Ocean; coast of Australia.

The organism defined here agrees closely with those described by Kofoid and Swezy and by Lebour. Distinctive characteristics are the slender form, twist of body, greater dorsal than ventral curvature, and a longer epicone than hypocone.

WARNOWIA Lindemann

Warnowia parva (Lohmann) Lindemann

(Plate 4, Figure 8)

Pouchetia parva Lohmann, 1908, p. 264, pl. 17, fig. 23.

Body elliptical, circular in cross-section, slightly tapered posteriorly, epicone somewhat larger than hypocone. Length 22.5–30 μ, width 15–18 μ. Epicone

hemispherical, hypocone similar but narrowed toward antapex and with sulcal indentation along left contour. Girdle shallow but very wide, displaced 0.50 the body length, with strongly descending right limb. Sulcus narrower than girdle, extending in sigmoid path from apex to antapex, bending from right margin toward center anterior to girdle, diagonal across ventral face between girdle ends, recurving from left margin toward center posterior to girdle. Anterior flagellar chamber an elongate pocket from left end of girdle limb, the posterior flagellar chamber not seen. Anterior flagellum not encircling body completely; posterior flagellum short, 0.33 body length.

Yellow bodies, resembling chromatophores in color and peripheral position but unlike in large size and irregular shape, sparse anteriorly but abundant posteriorly. Nucleus anterior, with elongate, oriented corpuscles. Melanosome near antapex, black, diffuse and spreading, or ellipsoidal and globular. Lens simple without evident laminations, projecting anteriorly on left side from melanosome. Nematocysts absent. Cell often within a pellice. Assimilate bodies frequent.

Woods Hole area: Great Pond; July. Baltic off Kiel.

The organism studied here is probably a close match to Lohmann's *Pouchetia parva,* but Lohmann showed no girdle and sulcus. It is close to *Nematodinium armatum,* but the tapered hypocone, large irregular chromatophores, absence of nematocysts, and cyst differentiate it, as well as the characteristics mentioned under *N. armatum.*

NEMATODINIUM Kofoid and Swezy

Nematodinium armatum (Dogiel) Lebour

(Plate 4, Figures 9, 10)

Pouchetia armata Dogiel, 1906, p. 36, pl. 2, figs. 48–49.
Nematodinium armatum (Dogiel) Lebour, 1925, p. 71, pl. 10, figs. 5a–5b
Nematodinium armatum (Dogiel) Lebour in Martin, 1929, p. 19, pl. 2, figs. 5–7.

Body elliptical, with equal epicone and hypocone. Length 33–53 μ, width 20–33 μ. Epicone evenly rounded, slightly asymmetric in ventral view, its right margin more sloping than the left; hypocone also slightly asymmetric with more sloping left than right side, the antapex either somewhat pointed and off-center, or, usually, obliquely truncate. Girdle deep, moderately wide, displaced 0.33 the body length, with strongly descending right limb. Sulcus extending from near apex in a sigmoid path to antapex, widening on the oblique margin of antapex, a portion of it curving sharply to the right, delimiting a knob-like protuberance on ventral face of the hypocone. Anterior flagellar chamber an elongate pocket from left end of girdle limb (posterior chamber not seen). Posterior flagellum one body length (anterior flagellum not fully studied).

Chromatophores yellow, circular or subcircular, few, and scattered. Nucleus large, anterior, of elongate chromatin corpuscles. Melanosome near antapex, black, diffuse and spreading, or ellipsoidal and globular. Lens single with concentric laminations, projecting anteriorly on left side from melanosome. Nematocysts present or absent, in region of right girdle limb.

Woods Hole area: Great Pond; August. Barnegat Bay, New Jersey; Plymouth Sound; Naples.

Some of the specimens were of full, ellipsoidal proportions, as in Dogiel's figure. Some were more slender, though not quite so slender as in Lebour's Figure 5a. Some had a slight tapering of hypocone and pointed antapex as in Martin's figures. They are all intermediate between the smaller *Warnowia parva* (Lohmann, 1908) without rightward curvature of sulcus on hypocone and with comparatively wider, shallower furrows, and the larger *Nematodinium lebourae* (Schiller, 1933; Kofoid and Swezy, 1921, as *N. armatum*), with rightward curvature of sulcus on hypocone and with comparatively greater transverse displacement of the sulcus.

POLYKRIKOS Bütschli

Key to Species

Chromatophores present:
- Body cylindrical ..... *P. hartmanni*
- Body laterally flattened ..... *P. lebourae*

Chromatophores absent ..... *P. schwartzi*

Polykrikos hartmanni Zimmermann

(Plate 4, Figure 7)

Polykrikos hartmanni Zimmermann, 1930, p. 436, figs. 8–9.

Colony cylindrical in form with rounded ends, consisting of two zooids, delimited by a slight constriction. Length 60–68 μ, width 42–47 μ. Epicone often smaller than hypocone in anterior zooid but equal to hypocone in posterior zooid. Girdles wide, rather shallow, displaced twice their width. Sulcus continuous from apex to antapex, roughly straight, narrowed at the constriction between zooids and in intercingular regions. Sulcus produced inward as anterior and posterior flagellar chambers, which extend posteriorly and anteriorly, respectively, their diverging ends overlapping. Anterior flagella incompletely encircling zooids; posterior flagella about 0.66 as long as the colony.

Chromatophores circular, small, numerous, yellow-brown in color. Nuclei always two, with elongate chromatin corpuscles. Several long nematocysts often, but not always, present in region just below anterior girdle. Innumerable black granules may fill peripheral cytoplasm of whole colony or may be restricted to posterior end.

Woods Hole area: Great Pond; August. Adriatic.

Slight differences between our specimens and Zimmermann's are the smaller size of ours (Zimmermann's 80–120 $\mu \times$ 55–75 μ) and the yellow-brown instead of yellow-green chromatophores. *Polykrikos barnegatensis* (Martin, 1929), also composed of two cells, is close to *P. hartmanni* but has a single nucleus and a more elliptical outline.

Polykrikos schwartzi Bütschli

Polykrikos schwartzi Bütschli in Kofoid and Swezy, 1921, p. 400, text fig. F, 4.
Polykrikos schwartzi Bütschli in Lebour, 1925, p. 67, pl. 10, figs. 2a–2b, text fig. 16c.

Woods Hole area: Great Pond; August. Arctic near Iceland; off coast of Norway; Skagerack; Baltic off coast of Denmark; Baltic off Kiel; North Sea off

Helgoland; Plymouth Sound; Atlantic off Concarneau, France; Mediterranean off French coast.

This species is distinguished by its many cells (zooids), averaging about eight, with four nuclei; by its cylindrical form; and by the absence of chromatophores.

Polykrikos lebourae E. C. Herdman

Polykrikos lebourae E. C. Herdman, 1924b, p. 60, fig. 6.
Polykrikos lebourae E. C. Herdman in Lebour, 1925, p. 68, pl. 10, fig. 3.

Woods Hole area: Salt Pond, beach sand; November. Port Erin, Ireland, in sand.

This species is quite distinctive in its lateral flattening. It has eight cells, two nuclei, and yellow-brown chromatophores. It is recorded from beach sand at Woods Hole by E. C. Herdman.

Diagnoses of New Species

Amphidinium **carteri** n. sp.

Corpus dorsali-ventraliter compressum, a fronte visum ovale; epicono minuto, asymmetrico et rostroformi; hypocono truncato-elliptico; sulco prope marginem dextrum, leniter curvato; chromatophoro uno, parietali, perforato, fulvo. Longitudo 12–15 μ, latitudo 8–9 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Amphidinium **wislouchi** n. sp.

Corpus simile *Amphidinio carteri* sed paullo majus; chromatophoris multis, ellipticis, paululum radiatim ordinatis. Longitudo 20–25 μ, latitudo 14–16.3 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gymnodinium **lazulum** n. sp.

Corpus globosum; epicono conico vel campaniformi; hypocono hemispherico vel campaniformi; sulco tenui; corpore sine striis; chromatophoris absentibus; cytoplasmate pellucido, subcaeruleo, saepe cum multis corporibus multi-coloratis. Longitudo 28–34 μ, latitudo 30–32 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gymnodinium **stellatum** n. sp.

Corpus lateraliter compressum; epicono quadrangulato et hypocono hemispherico; extremis cinguli 0.17 longitudinis corporis transpositis; sulco a vicinitate apicis ad cavernam profundam hypoconi extendente; corpore sine striis; chromatophoris absentibus; structura cytoplasmatis exterioris perspicue radiata. Longitudo 25–47 μ, latitudo 22–39 μ, crassitudo 13–25 μ. United States, in loco dicto Salt Pond, Barnstable County, Massachusetts.

Gymnodinium **striatissimum** n. sp.

Corpus globosum vel ellipticum; epicono et hypocono conico, aculeato, vel truncato; cingulo angusto, flexuram posterioram extremi sinistri habente; sulco a vicinitate apicis ad antapicem extendente; corpore cum striis, in hypocono pluribus quam in epicono ornato; chromatophoris absentibus. Longitudo 29–43 μ, latitudo 23–41 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **estuariale** n. sp.

Corpus ellipticum, paululum asymmetricum, margine dextro convexiore quam margine sinistro; epicono late conico vel sub-hemispherico; hypocono hemispherico; extremis cinguli 0.25–0.33 longitudinis corporis transpositis; sulco ad latus dextrum inter extrema cinguli multum deflecto; corpore sine striis; chromatophoris fulvis, 2–4, paulum intra peripheriam positis. Longitudo 11–16 μ, latitudo 9–12 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **aureolum** n. sp.

Corpus globosum; epicono et hypocono hemispherico vel conico; extremis cinguli 0.33 longitudinis corporis transpositis; sulco a vicinitate apicis ad antapicem extendente; corpore sine striis; chromatophoris multis, fulvis, plerumque omnibus plus minusve radiatim ordinatis. Longitudo 27–34 μ, latitudo 17–32 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **resplendens** n. sp.

Corpus late fusiforme, apice et antapice truncato, dorsaliventraliter compressum; extremis cinguli 0.20–0.25 longitudinis corporis transpositis; sulco in epicono augusto, tenui; ad latus dextrum curvato, in hypocono profundo et recto; corpore sine striis; chromatophoris multis, fulvis, radiatim ordinatis. Longitudo 36–62 μ, latitudo 32–48 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **uncatenum** n. sp.

Corpus lateraliter compressum, a late visum ellipticum vel quadrangulum; extremis cinguli 0.33 longitudinis corporis transpositis, parte dextra ad antapicem multum curvata; sulco in epicono tenui, ad sinistram prope extremitatem posteriorem cinguli deflecto; hypocono excavationem profundam antapicis praehente; corpore sine striis; chromatophoris luteo-fuscis, elongatis, radiatim ordinatis; cytoplasmate circa peripheriam structuram radiatam habente. Longitudo 40–54 μ, latitudo 28–33 μ. United States, in loco dicto Uncatena Island, Barnstable County, Massachusetts.

Gyrodinium **metum** n. sp.

Corpus paulum asymmetricum, latere dextro convexiore quam latere sinistro; epicono conico, multo latiore quam hypocono; hypocono truncato, multo longiore quam epicono; cingulo profundo, extremis cinguli 0.20–0.25 longitudinis corporis

transpositis; sulco S-curvato, in epicono absente, in hypocono lato; corpore sine striae; chromatophoris absentibus. Longitudo 14.5–2 μ, latitudo 11–16 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **glaebum** n. sp.

Corpus ellipticum, paulum asymmetricum; epicono hemispherico, hypocono late hemispherico; cingulo lato, extremis cinguli 2–3 latitudinibus cinguli transpositis; sulco in epiconum vix extendente, in hypocono lato et profundo; corpore sine striis; chromatophoris absentibus. Longitudo 17–25 μ, latitudo 12–19 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **undulans** n. sp.

Corpus ellipticum; epicono subhemispherico vel truncato-pyramidali; hypocono truncato-pyramidali; extremis cinguli 0.20 longitudinis corporis; sulco in duo S-formata curvamina facto, margine sinistro in hypocono marginem dextrum superposito; corpore sine striis; chromatophoris absentibus. Longitudo 27–38 μ, latitudo 21–31 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **dominans** n. sp.

Corpus fusiforme; epicono et hypocono conico; extremis cinguli 0.25–0.33 longitudinis corporis transpositis; sulco S-curvato, in epiconum extendente, in hypocono ad marginem sinistrum prope antapicem extendente; corpore cum striis, 7–10 a ventrale viso in epicono et hypocono; chromatophoris absentibus. Longitudo 18.5–43 μ, latitudo 10–22 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

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Summary

1. Unarmored Dinophyceae were collected from very shallow embayments on the south shore of Cape Cod, Massachusetts.

2. Twenty-six species, distributed in eight genera, were studied. Twelve were considered as new species and nine showed extensions of range from Europe.

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THE NATURE OF CERTAIN RED CELLS IN DROSOPHILA MELANOGASTER

JACK COLVARD JONES [1] AND E. B. LEWIS [2]

In a stock of the spineless (*ss*) mutant of *Drosophila melanogaster,* some of the flies were observed to have bright red cells under the cuticle. The presence of these pigmented cells has been found to depend upon a recessive mutant gene located at 26.0 ± in the second chromosome. The mutant has been named "red cells," symbol, *rc*. This paper is a brief account of the location, histology, and cytology of the red-pigmented cells of the *rc* mutant.

Methods

A stock homozygous for *rc* and *ss* has been used for all studies unless otherwise specified. The *ss* mutant serves merely as a marker to check on contamination of the stock and does not have any obvious effect on the expression of *rc*. Under crowded culture conditions *rc* may overlap wild type. To obtain maximum expression of the *rc* mutant, it is desirable to rear the larvae on an abundant supply of yeast. In the present work, additional dried yeast or paper towelling saturated with a thick fresh yeast suspension was added to the standard culture medium on the fourth day after introducing the parents. To study the effect of trypan blue, the *rc* mutant was grown on standard culture media containing 1.5% trypan blue.

Larvae of the third stage, pupae of various ages, and young adults of both sexes were studied. Intact larvae were immersed in 0.85% NaCl or in water, covered with a cover slip, and their various tissues examined *in situ* under high power (970 X).

Pupae and anaesthetized adults were pinned to a paraffin dish and dissected in Beadle-Ephrussi saline or in ethyl cellosolve. While a variety of fixatives were employed, cellosolve-fixed specimens were chiefly used. Fixed specimens were transferred to methyl benzoate and finally embedded in paraffin. Serial, cross, and sagittal sections were stained principally with picric acid since this stain did not interfere with the recognition of trypan blue uptake nor of red pigment distribution. Several series were stained with methylene blue. One series was stained with hematoxylin and eosin, two series with Sudan III for fat, and two series according to the Bauer test for polysaccharides.

Observations

Third-instars and early pupae of the *rc* strain, whether examined as whole mounts or as sectioned material, do not show any pigmented cells. In older pupae,

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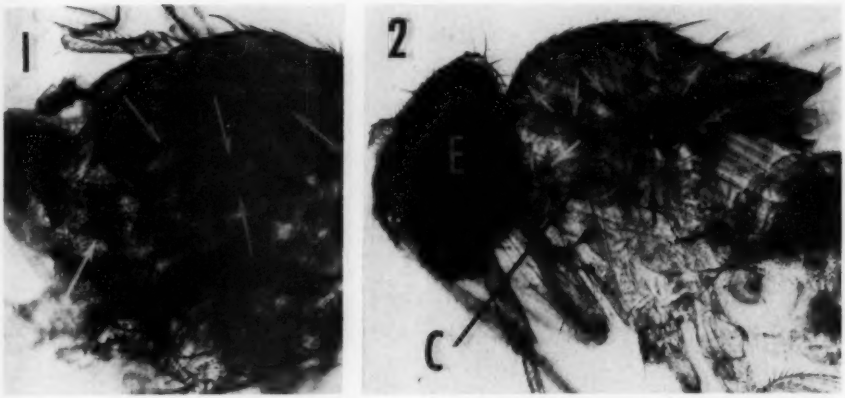

FIGURE 1. Dorsal view of thorax of adult *Drosophila rc* mutant showing alignment of red-pigmented cells. Unstained whole mount. × 87.

FIGURE 2. Lateral view of *rc* mutant showing clusters of pigmented cells. C = cardia, E = eye. Unstained whole mount. × 63.

a pale yellow substance appears in the eye and in many of the cells destined to contain red pigment granules. Red granules first appear in scattered cells in the head *before* becoming visible in the facet cells of the eye (Fig. 3). Whether yellow material is itself converted directly into red pigment has not been determined. As pigment in the eye changes from pale brown to dark brown and then to red brown, the number of red granules in the cells of the head and thorax increases from one or two to many per cell. Before the granules actually form, or when only one or two are present, these thoracic cells are conspicuous by their yellow tinge. By the time the eye pigment is formed, pigment is present in the cells of the head, thorax, and abdomen.

Living *rc* flies of both sexes show red-pigmented cells under the cuticle in the thorax and head (Figs. 1 and 2). While red-pigmented cells may be widely distributed throughout the body of young adults, they are most numerous and conspicuous in the head and thorax. In the thorax, loose aggregations occur in two longitudinal rows along the dorsal midline of the mesonotum and scutellum (Fig. 1), and there are less conspicuous groups on either side of these. Small clusters of pigmented cells are sometimes seen in the legs (coxa and trochanter) and isolated pigmented cells occur within the abdomen (Fig. 5).

The red-pigmented cells occur either singly or in definite groups of four or five or more, but they do not form syncytia (Fig. 4). When the cells are in definite locations, as in the supra-aortal masses and alongside the anterior part of the gut (Fig. 6), they are not bounded by connective tissue membrances and hence are extremely difficult to examine in fresh dissections.

The pigment granules are round, ovoid, or irregular in shape (Fig. 4) and they vary in size from less than 0.5 to about 4 microns. The number of granules per cell ranges from a few to 50 or more. The cells bearing the granules are round or ovoid (Figs. 3–6), measure about 20 microns in diameter, and have a single

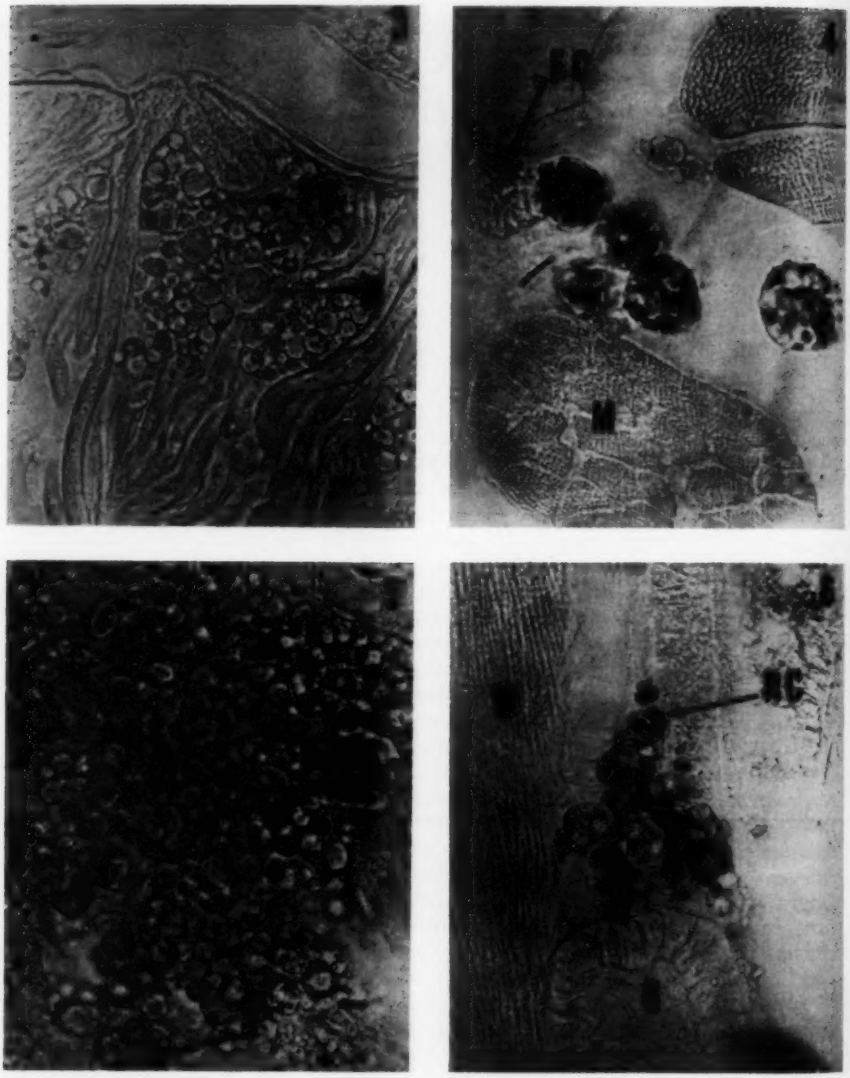

FIGURE 3. Horizontal section through head of young pupa before eye pigment has formed, showing a red pigmented fat cell (RC). Hemocyte is shown at H. Picric acid. ×940. Ref. no. 2173.

FIGURE 4. Typical red-pigmented cells in the thorax. Muscles are shown at M and an unpigmented fat cell at FC. Picric acid. ×1120. Ref. no. 2174.

FIGURE 5. Fat cells in the abdomen of an adult fly, showing a single cell with red granules at R. Picric acid. ×1120. Ref. no. 2172–5.

round nucleus. They are thus considerably larger than hemocytes which measure between 5 and 10 microns in diameter. The red cells, when examined in wet mounts, generally have large non-refringent droplets and other inclusions in their cytoplasm, in addition to pigment. Isolated pigment cells in saline do not send out lamellar extensions, and thus differ from certain kinds of hemocytes.

When larvae are reared on trypan blue media enriched with added yeast, the resulting pupae and adults have blue dye in the gut lumen, hemocytes, scattered thoracic fat cells, garland cells, and thoracic and abdominal nephrocytes. In the nephrocytes the dye appears in irregular diffuse granular masses and/or in dense aggregates. Sometimes large dye droplets are seen free in the hemocoele. Only occasionally do cells having red pigment take up the dye. Abdominal fat cells do not take up trypan blue.

Red-pigmented cells of the *rc* strain have large fat droplets (Sudan III) and smaller deposits of polysaccharide (Bauer-positive material), and are in the same size range as typical fat cells in the head and thorax. Fat cells in the abdomen of adults are distinctly larger (25–35 microns) than those in the head or thorax (14–21 microns).

Combinations of *rc* With Other Mutant Genes

The *rc* mutant was combined with mutants which affect the eye color in order to determine whether the red granules in the fat cells are related to the eye pigments. When *rc* is combined singly with mutants which remove the brown component of the eye pigment, namely, vermilion, cinnabar or scarlet, the homozygous double mutant combination has no pigment in the fat cells. When *rc* is combined with the brown mutant, which removes the red component and leaves the brown component of the eye pigment, the homozygous double mutant has typical red fat cells. It should be added that *rc* does not modify the eye color of wild-type nor of any of the above mutant types.

The *rc* mutant was combined with Microcephalus, a dominant mutant, locus 60.0 in the third chromosome which frequently results in a completely eyeless fly. Eyeless specimens thus obtained show the same degree of pigment development in the fat cells as do the cells of *rc* flies with normal eyes.

Discussion

The red-pigmented cells in *Drosophila* adults resemble typical peripheral fat body cells of the thorax and head in size, shape, general distribution, and possession of deposits of fat and polysaccharide.[3] However, they do not usually stain vitally with trypan blue, while some other typical head and thoracic fat cells without red pigment often incorporate the dye. Those relatively few red-pigmented cells present in the abdomen are usually peripheral in location and of about the same

[3] Dr. M. T. M. Rizki, who has examined our *rc* mutant, independently concludes from his observation of the red-pigmented cells that they are fat cells (personal communication).

Figure 6. Sagittal section through thorax of adult, showing clusters of cells lateral to the gut (G), some with trypan blue and others with red granules (RC). Picric acid. × 940. Ref. no. 2174–10.

size as the thoracic fat cells. Red fat cells are larger than typical hemocytes and do not send out lamellar extensions ("pseudopodia") in fresh dissections as many hemocytes do *in vitro*. None of the sessile or circulating hemocytes examined had red pigment. Indeed, so far as the authors are aware, there is no report of hemocytes with colored pigments among the insects.

Red pigment in fat cells first appears in the pupal stage at about the same time as pigment forms in the eyes. Combinations of *rc* with eye color mutants strongly suggest that the pigment in the fat cells is closely related to, or identical with, the brown eye pigment. Microscopically, brown eye pigment is red in color. That the pigment has not diffused out of the eyes and been secondarily taken up by fat cells is shown by the presence of abundant red fat cells in eyeless *rc* flies.

Summary

A mutant of *Drosophila melanogaster* possesses pigmented, stationary cells in the body cavity of the pupal and adult stages. The pigment is present as numerous red granules in the cytoplasm. By a number of criteria, the pigmented cells are a type of fat cell. The evidence suggests that the pigment is related to, or identical with, the brown component of the eye pigment and that it develops in the fat cells autonomously.

VASCULAR BUDDING, A NEW TYPE OF BUDDING IN BOTRYLLUS[1, 2]

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In the early days of the investigation on compound ascidians, it was believed that botryllids propagate either by stolonial budding alone or by stolonial *and* palleal (peribranchial) budding. It was Seeliger (1907) who, in his monumental treatise on ascidians, denied once and for all the occurrence of stolonial budding in botryllids. According to him, the supposed stolonial budding in these ascidians is palleal budding which was misinterpreted. After Seeliger, all the workers on ascidians agreed that the only budding in botryllids is palleal (*cf.* Huus, 1937; Brien, 1948; Berrill, 1950; *et al.*), except perhaps E. C. Herdman, who maintained as late as 1924 that in *Botryllus* buds are occasionally formed from the blood vessels of the test.

Since 1950 we often have had opportunities to observe that, in *Botryllus primigenus* at least, buds are formed from blood-cells gathered at the base of ampullae. It is proposed to name this type of budding "vascular" as distinct from "stolonial," for in *Botryllus* the blood vessels are generally called test vessels and not stolons, and, further, the buds are formed from blood-cells, and not from the mesenchymatous septum as in stolonial budding.

In this paper the process of vascular budding will be described in some detail and a comparison will be made between this and the ordinary palleal budding.

Historical[3]

Following are the various views concerning budding in botryllids, arranged in chronological order.

Savigny (1816), in his description of the marginal tubes (test vessels) in *Botryllus,* seems to have regarded them as an apparatus for the production of buds; this view, which was more fully elaborated and established by Milne-Edwards (1842), was generally adopted until Metschnikoff (1869) demonstrated that in *Botryllus* gemmation takes place from the sides of the ascidiozooids, *i.e.*, budding is palleal. This view of Metschnikoff was fully confirmed by later investigators such as Della Valle (1881), Oka (1892), *et al.*

Ganin (1870), however, maintained that the buds can be formed also "auf langen Stolonen und weit entfernt von dem Körper der Ascidien . . ." (p. 517).

[1] Contributions from the Shimoda Marine Biological Station, No. 92.

[2] The cost of this research has been partly defrayed from the Scientific Research Expenditure of the Department of Education of Japan.

[3] Because the wartime literature is only poorly represented in Japan, we consulted Prof. N. J. Berrill of McGill University, Montreal. He assured us in a letter that since 1940 no paper had been published on the stolonial budding in *Botryllus.* We wish to express here our thanks to Prof. Berrill for his kind information.

Giard (1872) also stated that in botryllids buds might be produced from blood vessels as well as from the body-walls of the ascidiozooids. In 1891, he still insisted that the inability of the test vessels to produce buds had not been sufficiently demonstrated.

W. A. Herdman (1886) described for *Sarcobotrylloides wyvillii* (and *Collela pedunculata*) that buds were produced intravascularly from aggregations of blood-cells. He went so far as to make the stolonial budding one of the characteristics of the family Botryllidae.

Bancroft (1903b) studied an aestivating colony of *Botrylloides gascoi* at Naples and found that many buds were formed in blood vessels apparently independently of zooids. He believed, however, that they were developed from zooids, not from blood vessels. He states (p. 151), "As no evidence in favor of an intravascular nor intra-ampullar origin of the isolated buds was detected, I feel convinced that they were developed from the zooids of the original colony before these had degenerated entirely. The buds must have severed their connections with the parent zooids, and must have been carried into the yellow lobe that was then being formed." According to the same author the buds described by Herdman (see above), too, might have been produced elsewhere and have migrated into the vessels.

Seeliger (1907) denied once and for all the occurrence of the stolonial budding in botryllids. According to him, the budding once described as stolonial is in reality typical palleal budding. As to how misinterpretation has come into existence, he says (p. 999): "Ich glaube, dass dieser Irrtum darauf zurückzuführen ist, dass die häufig in Rückbildung begriffenen Zooide, die bereits an Grösse abgenommen haben und mit den stoloähnlichen Mantelgefässen innig verwachsen sind, für neue Knospenanlagen gehalten wurden, die sich an und aus den erweiterten Gefässampullen entwickelt hätten."

E. C. Herdman (1924), however, could not reconcile herself to this view of Seeliger's and expressed herself in the following way (p. 4): "It is quite possible, however, that occasionally certain swollen knobs on the blood vessels of the test may give rise to buds." She adds, however, that "it is certainly not usual in *Botryllus* and there is no definite proof that it has ever occurred."

To sum up, it is today a well established fact that in botryllids buds are generally formed from the sides of ascidiozooids. Further, being organs for blood propulsion and respiration and perhaps also for excretion of test matrix, the ampullae are never transformed into new zooids. The problem is whether or not under certain circumstances buds are also formed from the walls of the blood vessels or the ampullae.

Materials and Methods

The following observations were made principally on living colonies of *Botryllus primigenus* Oka, occurring in the vicinity of the Shimoda Marine Biological Station, Shimoda, Japan. As is well known, the zooids in *Botryllus* are generally grouped into systems. In *Botryllus primigenus*, however, they are independent of one another, and each opens through its own atrial opening (*cf.* Oka, 1928).

To facilitate observation, colonies were fixed on glass slides. These, except at the time of observation, were set out in the bay. The technique employed for fixing the colonies was the same as that described in the paper of Oka and Usui (1944). The colonies grew well on glass slides.

Various developmental stages of the bud were observed and sketched under a binocular (magnification: × 72) and an ordinary microscope (magnification: × 100). Since the buds are formed from blood-cells, vital staining of these was tried with methylene blue and neutral red.

Observations on living materials were supplemented with examination of sections. In this case, strong Flemming's fluid was used as the fixative and the sections were stained with Heidenhain's haematoxylin and eosin. Staining with thionin (1% aqueous solution) was also tried.

All the observations were made at, or upon material obtained at, the Shimoda Marine Biological Station. It is our present duty to acknowledge our indebtedness to the Director and staff of that station for providing us facilities for carrying out this investigation. Thanks are also due to Miss Yoshiko Oshima who helped us in various ways.

Observations

Developmental cycle in the colony of *Botryllus*

In *Botryllus,* buds (palleal buds) appear very precociously, so that the constituting members of a colony are not single individuals, but aggregates of individuals belonging to three successive generations. They have been given the name "units" (Watanabe, 1953). Generally, a unit consists of more than three individuals. As a rule, two pairs of buds are formed by each individual, though not all of them develop. In the following text, as well as in Figure 1, each generation is represented by only one individual. An individual has a definite life-span. Its life has been divided into 11 developmental stages by Berrill (1941a).

A unit shows four different combinations of stages.

On the first day, a bud of stage 1 is seen on the lateral wall of a bud of stage 6, which in its turn is connected with a zooid of stage 9 by means of the connecting

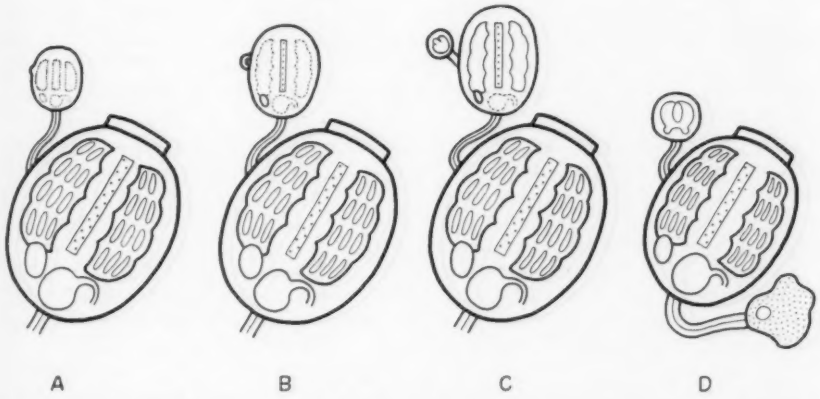


Figure 1. Developmental cycle of the unit in the colony of *Botryllus* (semi-diagrammatic). A, Phase A; B, Phase B; C, Phase C; D, Phase D.

vessel, *i.e.*, the colonial circulatory system. The combination of stages is 1–6–9. This phase is called phase A (Fig. 1, A).

On the second day, the bud of stage 1 has developed into a bud of stage 3. The bud of stage 6, on the other hand, has grown into a bud of stage 8. This latter is attached to the parent zooid which is still in stage 9. The combination of stages is 3–8–9. The phase is called phase B (Fig. 1, B).

Similarly, the phase on the third day is called phase C (combination of stages: 4–8–9) (Fig. 1, C), and that on the fourth day phase D (combination of stages: 5–9–11) (Fig. 1, D).

On the fifth day, the bud of stage 5 has grown into stage 6 with a new bud of stage 1 formed upon it. It is attached to the zooid of stage 9. The zooid of stage 11 has completely disappeared. The unit thus returns to phase A and starts a new cycle.

In each unit, the four phases are regularly repeated. Since all the units in a colony are exactly coordinated, we can also speak of the phases of the colony as a whole. A colony has four successive phases, which constitute a developmental cycle.

Formation of the vascular bud and its further development

The formation of the bud is initiated by gathering of particular blood-cells (diameter *ca.* 3–4 μ; see below) under the epidermis at the base of ampullae (Fig. 6). The number of cells is about 15–20. An intensive cell division follows, and, in one hour or so, a mass of cells (diameter *ca.* 20 μ) is formed (Figs. 2, 7). Since an ampulla is about 100–110 μ in diameter, the mass occupies about one-fifth of its width.

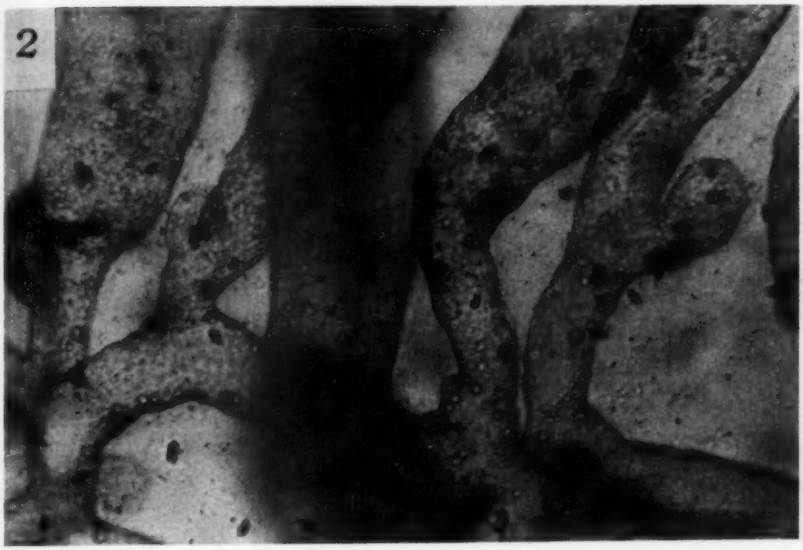
Active cell division continues, and, in two or three hours, a blastula-like structure is formed (Figs. 3, 8). At the same time, the side-wall of the ampulla that lies over the blastogenic mass begins gradually to protrude, and, in four to five hours, becomes distinctly visible as a bud (diameter 40–50 μ) (Figs. 4, 9).

At this stage, which will be designated stage 3, the anterior wall of the inner vesicle that faces the epidermis of the ampulla is two or three cells thick, while the remaining walls are very thin. Morphologically, the vascular bud of this stage exactly corresponds to the palleal bud of stage 3 (diameter *ca.* 48 μ) except that it has no ova.

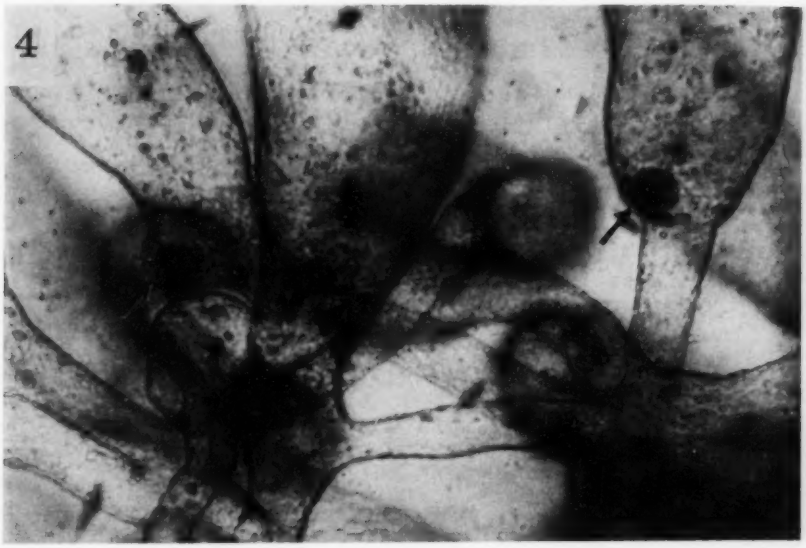
The cell layer constituting the outer vesicle or ectoderm of the bud is a direct continuation of the wall of the ampulla. The layer is at first closely applied to the inner vesicle, but later an ample space is formed between the two into which various kinds of blood-cells migrate, thus giving the impression that the inner vesicle is floating in the middle of the projecting part.

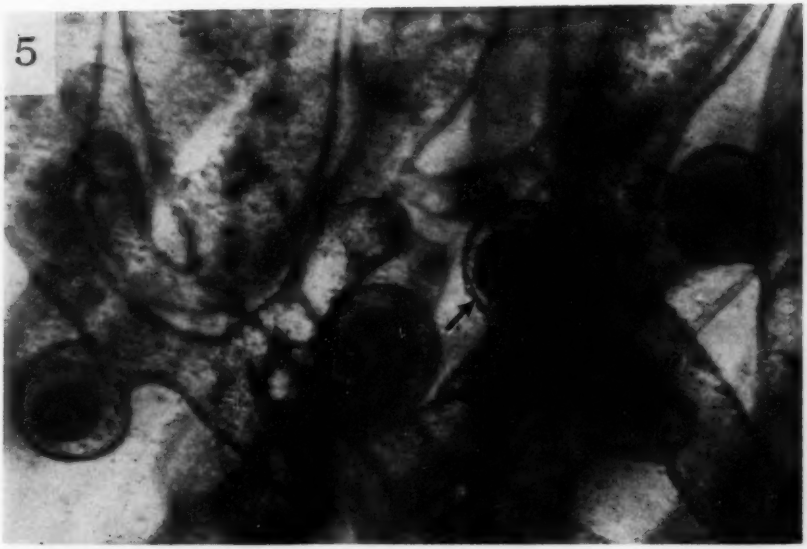
Development of the inner vesicle is as follows. As the anterior wall continues to expand, two vertical folds appear. These gradually extend backwards until they divide the vesicle into a median and two lateral chambers, which become later the central pharyngeal chamber and a pair of lateral atrial chambers. Next, three evaginations are formed, representing the heart, the neural mass and the intestine, respectively. Later development is primarily an elaboration of these unit regions.

Thus, the blastogenesis in vascular buds is an exact replica of that in palleal buds.

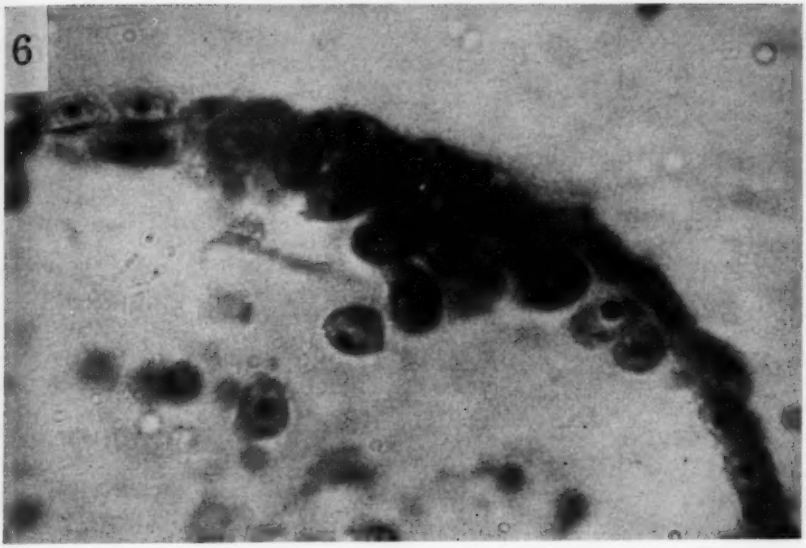


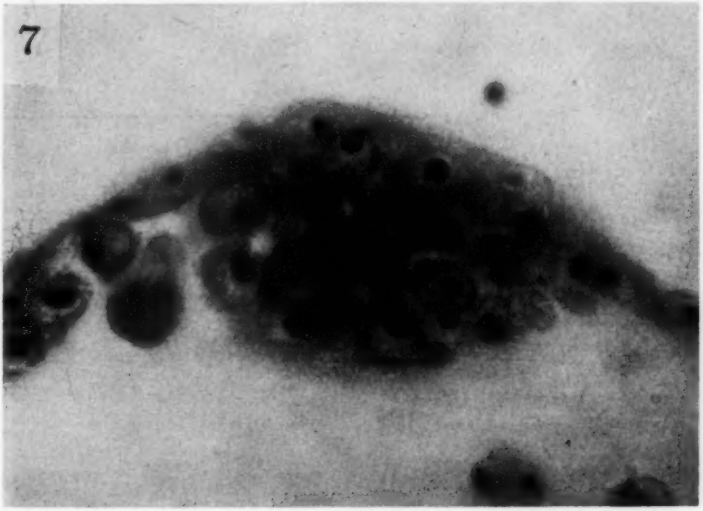
FIGURES 2-3. Development of the vascular buds. Figure 2 shows ampullae just before the appearance of vascular buds. × 120.



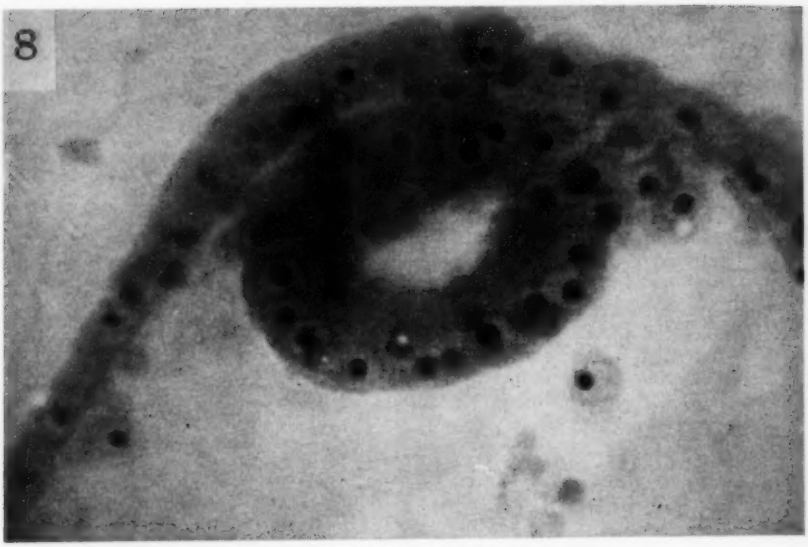


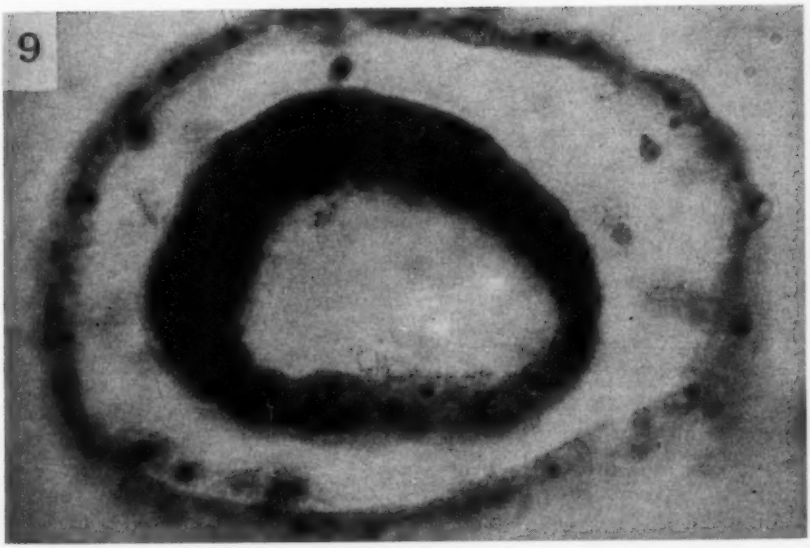
FIGURES 4–5. Development of the vascular buds (continued). × 120.





FIGURES 6–7. Development of the vascular buds; in sections. × 1200.





FIGURES 8–9. Development of the vascular buds; in sections (continued). Fig. 8, × 1200; Fig. 9, × 1000

Nature of the blood-cells

The blood-cells from which the inner vesicle originates are small, round cells. They show a strong affinity for thionin; further, they are more strongly stained by such vital stains as methylene blue (1/10,000 aq. sol.) or neutral red (1/10,000 aq. sol.) than other blood elements.

In sections, it is seen that the cells have a hyaline cytoplasm; their nucleus is filled with a dense network of chromatin and lacks a nucleolus. The total picture suggests that they are cells of a primitive nature. They are the lymphocytes in the terminology of Sabbadin (1955).

Time of appearance

The appearance of the vascular buds is limited to a certain phase in the life cycle of the colony. They appear only during a short period (about 10 hours), extending from later phase B to early phase C. At that time, the colony is at the maximum of its activity and, accordingly, the growth of the ampullae is also very active.

In other phases, no buds are formed even where they are expected to appear. In phase D we sometimes find small buds; however, they are not new buds but older ones that have appeared in early phase C and are now on the way to dissolution.

Site of appearance

In *Botryllus* colonies, numerous blood vessels traverse the test and terminate in contractile ampullae at the periphery of the colony; a flow of blood is maintained by them independently of heart action. The buds appear at the base of ampullae at a distance of about 0.6–0.7 mm. from the tip (Fig. 10).

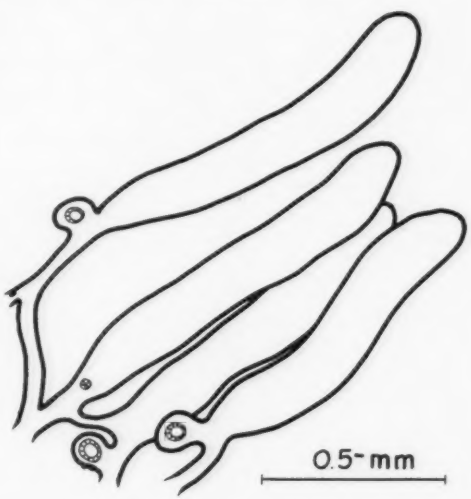


FIGURE 10. Vascular buds at the end of budding period. Note the various sizes.

On each ampulla, only one bud is formed at a time.

Not a single case has been recorded in which the buds are formed from the side-wall of the ordinary vessels.

So far as is known, the appearance of the vascular buds is restricted to the most actively growing edges of the colony where the ampullae are especially numerous and active.

Degeneration of the buds

At the end of the budding period (early phase C) we often find tens and hundreds of newly formed buds, but not all of them continue to develop. Only those which surpass a certain size can develop and form perfect zooids, while the remaining ones undergo involution without even approximately reaching the stage when the two vertical folds appear. For example, in a case out of 200 newly formed buds, only 70 developed into perfect ascidiozooids.

Table I shows an example of different sizes of the buds in a colony at the end of the budding period.

Of these 36 buds, those larger than 40 μ continued to develop (20 buds, or 56%), while those under 35 μ soon began involution and disappeared completely (16 buds, or 44%).

Relation between palleal and vascular budding

As is shown in Table II, the palleal bud appears in phase A. The vascular bud appears, as has already been pointed out, at the end of phase B, and attains stage 3 in phase C. It grows rapidly and becomes stage 5′ (a stage a little behind 5) in phase D and stage 6′ (a stage a little behind 6) in phase A of the following cycle. In phase B the vascular bud attains the stage 8, and from this moment on it develops exactly synchronously with the corresponding palleal bud. It also dies synchronously with the latter.

In brief, the life of the zooid produced by palleal budding extends over 12 days, while that of the zooids produced by vascular budding lasts for ten days. The development, especially in its early stages, progresses a little more rapidly in the vascular than in the palleal bud.

When the vascular bud has attained stage 6′ we see buds of stage 1 appearing on its peribranchial walls. Thus the vascular bud, once formed, propagates by palleal budding.

TABLE I

Various sizes of vascular buds at the end of the budding period

Diameter (in μ)	Number of buds
ca. 70	3
ca. 50	10
ca. 40	7
ca. 35	4
ca. 30	8
ca. 20	4
Total	36

TABLE II

Relation between palleal and vascular budding. Vascular buds are printed in bold-face type

Day	Cycle	Phase	Palleal budding	Vascular budding		
I		A	1			
II	n	B	3			
III		C	4	**3**		
IV		D	5	**5′**		
V		A	1–6	**1–6′**		
VI	n 1	B	3–8	**3–8**		
VII		C	4–8	**4–8**	**3**	
VIII		D	5–9	**5–9**	**5′**	
IX		A	1–6–9	**1–6–9**	**1–6′**	
X	n 2	B	3–8–9	**3–8–9**	**3–8**	
XI		C	4–8–9	**4–8–9**	**4–8**	**3**
XII		D	5–9–11	**5–9–11**	**5–9**	**5′**

Each vertical column represents the development of the same individual.

In the growing edges of a colony, the test vessels grow out continuously. The distal end of the ampulla grows out and forms the new tip, while its basal part is continuously transformed into the ordinary vessel. This means that the vascular bud, though first formed at the base of the ampulla, is gradually removed from the latter. By the time the bud has attained stage 4–8, it is already at some distance from the ampulla. At the same time we see that a new vascular bud is being formed at the base of the ampulla (Fig. 11). Thus an ampulla forms only one bud at a time, yet it can produce many consecutively.

As the vascular bud is formed a little later than the corresponding palleal one, it is at first smaller than the latter. At the end of development, however, no difference in size is perceptible between the two.

The full-grown zooids formed by vascular budding are the same as those produced by palleal budding in almost every respect, even in the number of tentacles

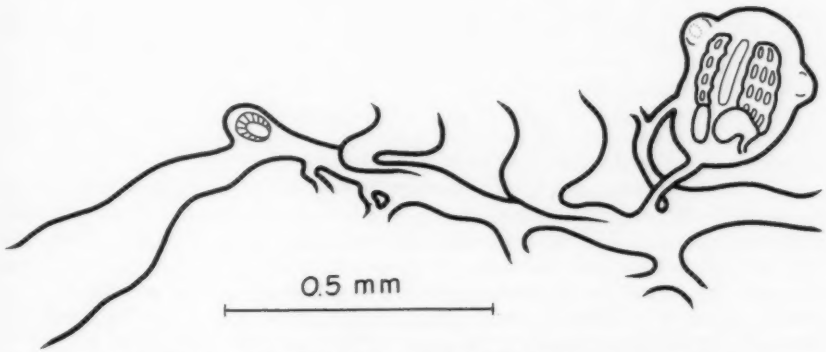


FIGURE 11. Two buds formed consecutively on the same ampulla.

or rows of stigmata. The only difference is that in vascular buds no gonads are formed. In palleal buds, the gonads segregate as a mass from the lateral walls of the bud at an extremely precocious period. Such a segregation has never been observed in vascular buds.

Discussion

Critical review of the earlier data

With this fully established vascular budding at hand, a critical review of the earlier data concerning budding in botryllids will be attempted.

Some of the earliest descriptions of the stolonial budding in *Botryllus,* such as those of Savigny or Milne-Edward, might have been founded on erroneous observations, but what Herdman observed in *Sarcobotrylloides wyvillii* and Bancroft in *Botrylloides gascoi* was in all probability vascular budding analogous to that described here. Some passages in Giard's paper (1872, p. 573) also make it probable that he really observed vascular budding in *Botryllus.*

According to W. H. Herdman (1886; pp. 59, 90), the buds were formed from three sources: large cells which became ova, blood corpuscles, and the wall of blood vessels. In the case of our buds, large cells which were to become ova could not be detected in the mass of cells.

The buds which were found in *Botrylloides gascoi* during aestivation, and which were entirely independent of parent zooids, are in our opinion certainly vascular buds, and not palleal buds developed from the zooids of the original colony and later carried into the yellow lobe, as was assumed by Bancroft. Bancroft met with the so-called yellow lobe only once, so he had no idea of its significance at the time when this was developing. He says (1903b, p. 152), "It is to be hoped that some future investigator will preserve and section the early stages of the development of the yellow lobe before the degeneration of the rest of the colony, and discover why in this case the buds separate from the parent zooids at such an early stage." These buds in all probability were formed *in situ* from blood cells.

There are some differences between our buds and those of Bancroft.

a) The buds described by Bancroft were formed after all the zooids had died away. Our vascular buds, on the contrary, are formed in an active colony simultaneously with palleal buds. In passing, it may be noted that in our opinion degeneration of zooids in the case of Bancroft was not a phenomenon of aestivation, but was caused by some unfavorable conditions in the aquarium.

b) The second difference is that in the case of Bancroft's material the usual coordination was entirely lost and the buds appeared so irregularly that it was practically impossible to tell to which generation they belonged, while our buds always appeared at the end of phase B of the colony.

c) The third difference is that the buds of Bancroft were scattered all through the colony, while ours are located strictly at the bases of ampullae. The examples of Bancroft perhaps show that the epithelial wall of the vascular system can develop into the ectoderm of the new buds, wherever these are formed. Why is it that normally the formation of the buds is localized to the base of ampullae? It is because the flow of blood is most sluggish there, thus giving the blood-cells the best opportunity for settling.

Vascular budding in the system of budding of ascidians

Whatever the type of budding may be, the initial stage of blastogenesis is a double-walled vesicle. The outer wall, called ectoblast, forms the epidermis, while the inner wall, called endoblast, forms the rest of the new zooid. The ectoblast invariably derives from the epidermis of the parent zooid, while the endoblast is variable in origin. According to this origin, the budding of ascidians is divided into three main groups: ectodermic, endodermic and mesoblastic. Vascular budding as described here, together with stolonial budding, belongs to the last group. Of all known cases of stolonial budding, that of *Perophora* comes nearest to the vascular budding of *Botryllus*. In *Perophora* the endoblast is formed from the mesenchymatous septum of the stolon.

Blood-cells in relation to budding

Generally, blood-cells are considered to have nothing to do with bud formation. Berrill (1951), for example, in a survey on regeneration and budding in ascidians states: (p. 468): "Trophocytes, 'cell-packets,' and the various blood cells, other than those lymphotic cells which at times arise from septal mesenchyme or the epicardial epithelium, apparently play no part in any morphogenetic or histogenetic processes, except as victuallers." Neither epicardium nor mesenchymatous septum does exist in *Botryllus*. But, since the mesenchymatous septum in other forms, notably in *Clavelina*, is known to gain or lose cells from or to the haemocoele as lymphochytes, the blood-cells of *Botryllus* forming new buds may possibly be conceived as mesenchymatous septum dissolved into its cellular elements and circulating with the blood stream.

According to the recent investigation of Sabbadin (1955), the blood-cells of *Botryllus* are of dual origin. One evolutionary series begins with a hemoblast. It has a vascular nucleus with a small amount of chromatin and cytoplasm rich in RNA-proteins. The other series begins with a lymphocyte. This is half the size of a hemoblast and is formed from the latter by division. Its nucleus is filled with a dense network of chromatin and lacks a nucleolus. Both series have their own leucocytes and vacuolated cells.

As is clear from the foregoing descriptions, the blood-cells partaking in the formation of vascular buds are lymphocytes as defined by Sabbadin. It seems rather strange that buds are formed from lymphocytes, not from hemoblasts which seem to have a still greater evolutionary capacity.

Size and morphogenesis

The relation between size and morphogenesis in palleal buds has been studied by Berrill (1941b). The palleal bud appears first as a thickened disc of atrial epithelium, which rapidly transforms into a closed sphere surrounded by epidermis. The new organism is formed by a process of folding and local evaginations of this expanding sphere.

Now there is seen a considerable variability in the size of the initial disc, and this determines, according to Berrill, the size and fate of the future zooid. Buds formed on too small a scale may fail to develop. Relatively small bud rudiments give rise to small zooids without gonads and with about six rows of stigmata, while

large rudiments form a greater number of rows of stigmata and produce testes and ripening ova; between these two there are many intermediate forms. In general, discs and spheres produced by early generations are relatively small compared with those produced by later generations. According to Berrill this accounts for the fact that juvenile colonies are asexual, somewhat older colonies have well-developed testes but no ova, and only at the last do both testes and ova appear.

The same explanation does not apply to the case of vascular buds. As has been demonstrated, the period during which the vascular buds appear is relatively short, extending from later phase B to early phase C. At the end of this period, we see buds of various sizes, but only those which surpass a certain size continue to develop, all the remainder degenerating.

We have studied in sections the number of blood-cells taking part in the formation of buds and found that variation is rather slight. The initial number of blood-cells, therefore, cannot be the cause of different sizes. The cause of the small size of some buds is to be sought in the belatedness of their appearance. Thus, small buds seem to have as full developmental capacity as larger ones. Their degeneration must, therefore, be understood as a manifestation of regulation of the colony as a whole.

The absence of gonads in vascular buds is also not the sequence of small size, for the average size of vascular buds is of the same order as that of palleal buds, in which well-developed testes and ova appear.

Regulating mechanism

Of all colony-forming animals, *Botryllus* is perhaps the one in which the zooids are most perfectly coordinated. We still know too little of the nature of the regulating mechanism, yet the fact that such a mechanism is working can be inferred from the following facts:

a) In a colony, the zooids are exactly coordinated in budding and development.

b) When two pieces of related colonies at different developmental phases are fused together, this difference is invariably equalized. This was first demonstrated by Bancroft (1903a) and is now being extensively studied by one of us (*cf.* Watanabe, 1953).

c) The vascular buds are formed a little later than the corresponding palleal buds, but they are soon synchronized with the latter.

d) Vascular buds formed too late are forced to degenerate, thus being eliminated from the colony.

Vascular budding in relation to the growth of the colony

In *Botryllus primigenus*, palleal and vascular budding coexist in a colony. Maintenance and multiplication of zooids are brought about by palleal budding, while localized, rapid growth of the colony seeking a new substratum is effected exclusively by vascular budding.

The colony can continue its existence by vascular budding alone. Such a state is realized when a piece of a colony containing no zooids but ampullae is isolated, or when all the zooids are experimentally removed, or again when, as in the case of Bancroft's material, all the zooids have died, owing to some unfavorable external

conditions. According to Bancroft (1899, p. 451) an isolated piece devoid of zooids never regenerated a colony, and none of the ampullae in such a piece showed the least tendency towards budding. On this point, therefore, we are quite at variance with him.

Significance of our discovery for the general theory of budding in ascidians

Our discovery is of significance in the following points:

a) In Stolidobranchiata, to which the genus *Botryllus* belongs, palleal budding has been considered the only way of asexual reproduction. We now know that *Botryllus* propagates also by vascular budding. This indicates that budding of a rather primitive nature, as the vascular, has not completely disappeared even in such a highly specialized group as Stolidobranchiata.

b) It is generally admitted that each species is given a single mode of asexual reproduction. Now, *Botryllus* propagates by two entirely different kinds of propagation, one ectodermic-peribranchial and one mesoblastic-vascular.

c) The lymphocytes are capable themselves of organizing new individuals.

Is vascular budding peculiar to Botryllus primigenus?

If we consider that of all the genera of compound ascidians it is *Botryllus* that has been most extensively studied till now, it is almost inconceivable that so typical a budding as the vascular has escaped the eyes of previous investigators. It is possible, although not very probable, that this type of budding is peculiar to our Japanese species. We have observed the same budding also in *Botryllus communis*, another Japanese species, which, however, in our opinion is homospecific with *B. primigenus*.

Summary

1. In *Botryllus primigenus*, it has been found that, in addition to palleal budding, new buds are formed also from aggregations of blood-cells at the base of ampullae.

2. The blood-cells partaking in the formation of buds are lymphocytes as defined by Sabbadin.

3. The formation of buds is possible only at a certain phase in the developmental cycle of the colony.

4. Even then, the buds are formed, not on all ampullae, but only on those lying in the most vigorously growing edges of the colony.

5. Our discovery is of significance in the following three points:

a. The ability to form new buds from the vascular wall has not completely disappeared in Stolidobranchiata.

b. A species can propagate by two entirely different kinds of budding. In our case, one is ectodermic-palleal and one mesoblastic-vascular.

c. The lymphocytes are themselves capable of organizing new individuals.

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OPTOKINETIC TESTING OF CYCLOPEAN AND SYNOPHTHALMIC FISH HATCHLINGS [1]

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Previous work on *Fundulus heteroclitus* embryos that were perfect cyclopeans, owing to treatment with ethyl alcohol, indicated that there is a strong tendency for the right side of the eye to function developmentally as a right eye would, in sending optic fibers to the left side of the brain, and for the left side of the eye to send fibers to the right side of the brain (Rogers, 1952). It seemed of interest to determine whether the right side of the cyclopean eye functions physiologically as a right eye would, and the left side, as a left eye. Optokinetic drum testing of optically abnormal amphibia (Sperry, 1944) suggested a means of making such tests. Even if positive optokinetic drum results were obtained with perfect cyclopean fish, it would be impossible to eliminate only one side of the retina surgically without damage to the other, but it might be possible to ablate one optic tectum to learn something further of the pathways involved. Nerve-stained sections could then be used to correlate the microscopic anatomy with the physiological results.

Pearcy and Koppanyi (1924) moved the left eye of a large goldfish to an artificial orbit in the top of the cranium, just to the left of the midline, without severing the nerve. The effect on equilibrium was noted and vision tested during four weeks. They stated that they had produced a real "experimental cyclops." This eye, however, has few of the characteristics of the cyclopean eye. It is not formed from the medullary plate material on both sides of the midline, it is not in the typical ventral position, and it did not develop its nerve connections while it was in close relation to the diencephalic floor on both sides of the midline.

Stockard (1909, p. 285) says in regard to cyclopean *Fundulus heteroclitus* treated with magnesium chloride solutions in the early stages: "Many embryos, showing the cyclopean defect in various degrees, hatched normally and were capable of swimming in a manner indistinguishable from ordinary two-eyed fish. These monsters gave many indications of ability to see. They went to the more brilliantly lighted side of the dish with the normal ones. They darted away in normal fashion when any object was placed in front of the eye, while similar objects put at equal distances from their tails caused no excitement." There appear to be no other reports in the literature of attempts to test cyclopean individuals for vision.

Materials and Methods

In the 1955 season 16,987 *Fundulus heteroclitus* eggs that successfully passed the early cleavage stages were treated with magnesium chloride solutions. Of

[1] This work was supported by Public Health Service grants, No. B–760 and No. B–760(C), and in 1955 by aid from the Committee on Productive Work, Oberlin College.

11,912 embryos surviving at least until the eyes were well formed, 0.3% were perfect cyclopeans with an eye of close to normal size, and only three such embryos hatched (Rogers, 1956). In the 1956 season 190 bowls containing somewhat larger numbers of eggs than in the 1955 work were treated in the same manner with magnesium chloride. An estimate was made that about 25,000 embryos survived until the eyes were well formed, 76 embryos (about 0.3%) were perfect cyclopeans with an eye close to normal size, and again only three such embryos hatched and swam. In 1955 the effect of temperatures between 11° and 16° C. was not tested. In 1956 water in bowls kept on the water table between June 6th and 12th was never below 15° or above 16° C. At this temperature seven bowls of 19/60 *M* $MgCl_2$ solution, with a total of 1,020 eggs that were living when they reached the blastula stage, produced 7% optic abnormalities among the 560 embryos that survived until the eyes were formed. All 12 cyclopean embryos among them had reduced eyes and stunted bodies. Although the mortality rate rose to 45%, as compared with 20% in the 1955 series at 18° C., production of optic abnormalities was increased by only 1%.

In the material just described, three cyclopeans, three synophthalmics, and one anophthalmic in the 1955 series, and three cyclopeans and one synophthalmic in the 1956 series hatched and swam well enough to be tested in the optokinetic drum. In addition, from batches of *Lucania parva* eggs fertilized with *Fundulus heteroclitus* sperm, one perfect cyclopean in 1955, and another in 1956, hatched and were tested. At first, a very large striped drum was tried, mounted on a horizontally-placed bicycle wheel revolving around a stationary central platform to hold the dishes of fish. Normal adults responded only moderately well, and hatchlings, not at all. Feeling that the hatchlings might not be affected by movement at such a distance, Mr. Michael Baron, helping with the technical work in 1955, lined an oatmeal carton only 10.5 cm. in diameter with vertical black and white stripes of one-half centimeter to one centimeter in width. When this drum was turned smoothly by hand around a small beaker containing normal hatchlings, the fish readily turned with the drum and quickly reversed directions when the drum was reversed. The fish were kept 6 to 10 days after hatching, and tested a number of times. When most of their yolk was used up, selected ones were drawn with camera lucida while under urethane anaesthesia, and all were fixed, sectioned, and stained with a modification of the Bodian protargol stain in the manner previously described (Rogers, 1952).

Results of Optokinetic Tests

Synophthalmic fish 89–1 (bowl number—individual fish number) and 89–2 (Fig. 4) responded directionally to drum rotation in the same manner as normal control hatchlings. Fish 89–1 did not start to turn with the drum as readily, and did not turn as rapidly as control fish, but otherwise exhibited normal optokinetic reflexes. Fish 89–2 responded nearly as well as controls. Synophthalmic fish 341–1, similar in appearance to the preceding two, did not respond to the drum, although it frequently swam at times when there were no apparent external stimuli. The unusual synophthalmic fish with right eye much reduced, 76–5 (Fig. 5), was slightly but permanently flexed toward its left. It did not orient well and often lay on its back on the bottom of the dish. Nevertheless, in any orientation, it

could always be made to flex vigorously and repeatedly toward its own left with drum rotation in the *opposite* direction, whereas no movement could be elicited by drum rotation to its left. If nerve connections of such an eye are contralateral, as they normally are, the animal ought to flex or turn readily in the same direction as the drum when the drum is rotated toward the blind side, and turn hesitantly or not at all when the drum is rotated toward the seeing side.

The cyclopean "magnesium" fish 89–3, 105–1 (Fig. 1), 105–2, 340–2, 340–3, and 360–1, the cyclopean *Lucania-Fundulus* hybrids 263–1 (Figs. 2 and 3) and 478–1, and the anophthalmic "magnesium" fish 109–1 all failed to respond to any drum stimulation tried. Various tests were made on all these fish, but hatchling 105–1 was tested more extensively than the others, over a period of five days. Drums of various sizes with various widths of striping and turned at various speeds under various lighting conditions were used. The fish were sometimes positioned very close to the beaker side and the drum rotated so that its surface passed just outside the glass and very near the fish. Black-and-white, and red-and-white stripes, striped cones revolving under as well as around the beaker, and flat discs with alternate opaque cardboard and cut-away strips radiating from a central hub and rotated over a light bulb with the fish just above the disc, were also tried with uniformly negative results. Any movements on the part of the fish appeared to be fortuitous and unrelated to the drum rotation. When testing was not being carried on, the anophthalmic 109–1 appeared to swim around the dish at more frequent intervals than normal control fish.

The failure of the perfect cyclopean fish to respond to the drum in 1955 led to the second season's work with the hope of obtaining positive results or of making the negative results more certain. The lack of response also made of prime importance the question as to whether the perfect cyclopean fish can see at all. Unfortunately, all tests so far tried have failed to give rigorous proof. "Baiting" the fish from outside a glass container (Sperry, 1949) appears to be impossible with these hatchlings. Numerous attempts to observe the effect of a differentially lighted container (Stockard, 1909) failed to yield unequivocal results in the present work. The chromatophore response to visually perceived light in adult *Fundulus* (Butcher, 1938) was considered, but light does not have an obvious effect on the chromatophores of normal hatchlings. Potential-recording methods seem impractical with these small fish. Fish 89–3 was placed in a glass container within the large drum mounted on the bicycle wheel, and the drum rotated for five-minute periods with three- to four-inch-wide black-and-white stripes visible, and then for similar periods with the stripes covered by a blank white paper. The number of times the fish swam, and the total length of time in seconds it was swimming, were recorded. A number of tests made it clear that these data were too variable to afford rigorous proof of ability to see. The only evidence obtained consisted of the apparent ability of perfect cyclopeans 89–3, 105–1, and 105–2 to localize the tip of a fine forceps when it was placed in front of them. These observations were not considered conclusive and therefore no attempt was made to repeat them on the perfect cyclopeans of the second season. Fish 105–1 responded 5 or 6 times in succession to a forceps placed close in front of it, by jerking quickly away. On the two succeeding tries, however, the fish slowly and deliberately came off the bottom and moved forward, accurately placing its tubular mouth against the forceps.

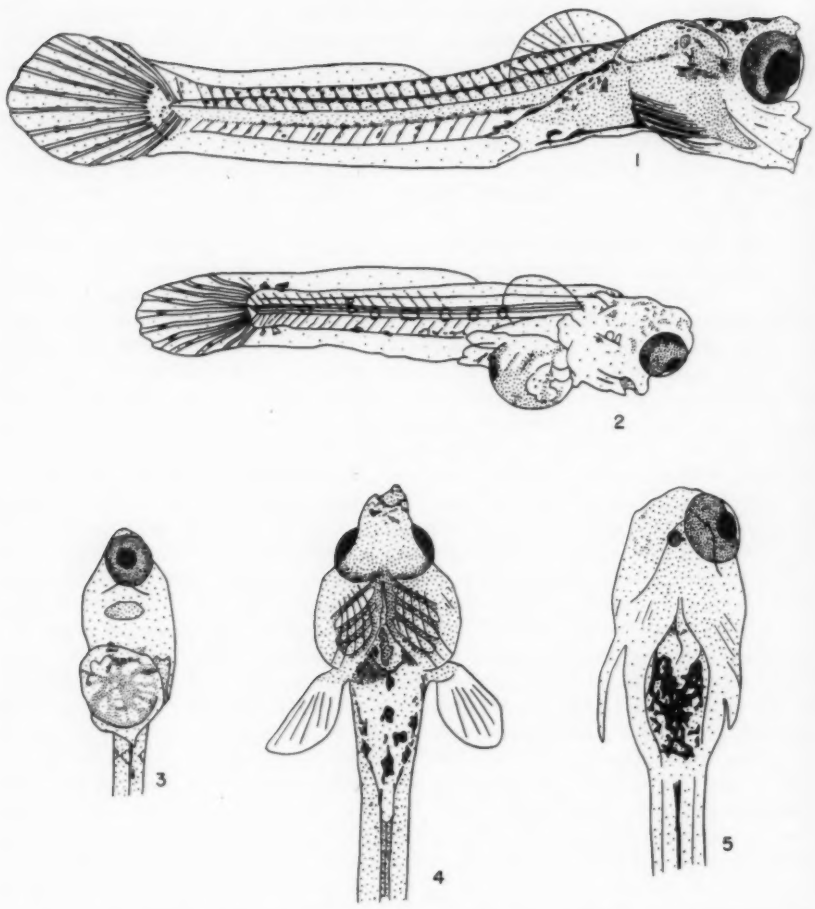


FIGURE 1. View of right side of cyclopean *Fundulus heteroclitus* hatchling 105–1. The anterior part of the body is flexed slightly to the right to show the eye. Drawn with camera lucida, urethane anaesthesia, × 20.

FIGURE 2. View of right side of cyclopean *Lucania parva—Fundulus heteroclitus* 263–1. Chromatophores are opaquely white rather than black as in *Fundulus*. × 20.

FIGURE 3. Ventral view of anterior part of 263–1, × 20.

FIGURE 4. Ventral view of anterior part of synophthalmic *Fundulus heteroclitus* 89–2, × 20.

FIGURE 5. Ventral view of anterior part of *Fundulus heteroclitus* 76–5, × 20.

It ignored the forceps when the latter was placed the same distance from its tail. When fish 105–2 had exhausted its yolk and become fairly inactive, it repeatedly exhibited following eye movements in the proper direction as a forceps tip was

moved across its front. Fish 89–3 responded to the forceps in front of it by moving away. All of these responses could conceivably be initiated by movement of water created by the forceps, but particularly in the case of the immobile forceps that was approached and accurately localized by fish 105–1, visual cues would seem to be a more likely explanation.

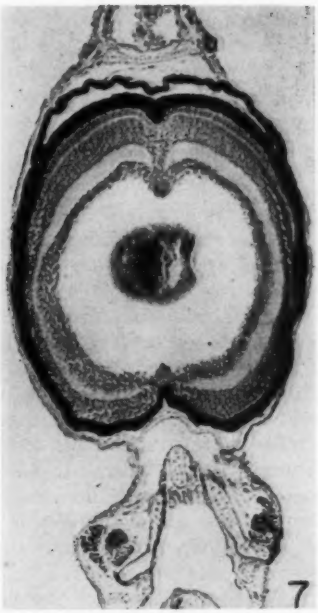
Histological Correlations

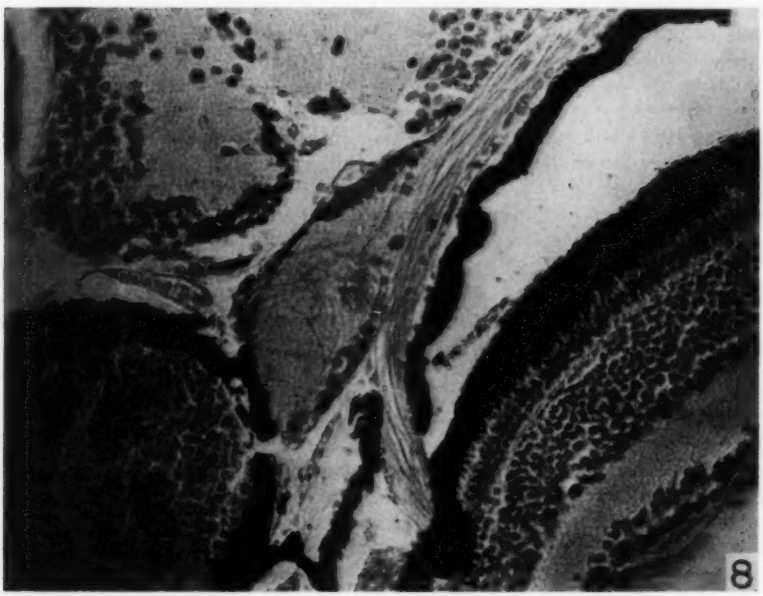
Synophthalmic fish 89–1, 89–2, and 341–1 each have two pupils and lenses, two arcs of sensory retina and pigment epithelium, and nerve bundles collecting from each retinal arc and passing out from the retina medially between the two arcs. Fish 341–1 has two choroid sacs and lacks an extensive fused area between the eyes, but 89–1 and 89–2 each have a single choroid sac enclosing the retinas, and a ventro-medial continuity of the sensory retinas (Fig. 6). The optic nerves in 89–1 and 89–2 pass posteriorly through the choroid coat in a single bundle and, possibly with a complete decussation, enter the floor of the diencephalon and proceed dorsally in two fairly equal bundles to distribute to the two optic tecta. Nerve fibers are so numerous, and they run parallel so far before turning up into the brain, that it is difficult to follow specific groups of fibers certainly enough to be sure there is complete decussation. The optic nerve in 341–1 collects from the retinal arcs and leaves the eyes in much the same manner, but after entering the diencephalic floor, it turns abruptly anteriorly and distributes to the telencephalon on the right side only. Fish 76–5* has a left eye that is fairly normal in form but that is displaced antero-medially over a cyclopean-type tubular mouth. The right eye consists of a small mass of sensory retinal cells, completely surrounded by a layer of pigment epithelium, and lacking a nerve. The left eye sends a large nerve entirely to the left side of the brain (Fig. 8). Part of this nerve passes into the left optic tectum and part passes anteriorly to distribute in the dorsal part of the diencephalon on the left side.

The form of the cyclopean eye is well known (Fig. 7). The eyes of perfect cyclopeans 105–1 and 105–2 each have a stalk similar to that of a normal eye. In both these fish, nerve fibers collect from both sides of the retina, leave the eye posteriorly, just behind a ventral retinal gap interpreted as the remains of a choroid fissure, run together in the optic nerve, sort out in the base of the diencephalon with a possible decussation, and distribute about equally to the two tecta.

Cyclopean fish 89–3 has no optic stalk, but instead the eye is continuous with the diencephalic floor over a wide area, the pigment epithelium appearing as a reflection of the diencephalic surface. The optic nerve collects from both sides of the retina but goes only to the right tectum. The eyes of the two hybrid cyclopeans, 263–1 and 478–1, have the same close relation to the diencephalon, with the stalk lacking, but in these cases the nerve distributes to both tecta. In 263–1 three-fourths of the optic fibers go to the left tectum and the remainder to the right. As they enter the diencephalon, some of these fibers certainly decussate but others do not. In 478–1 the fibers collect in a bundle and run parallel to each other for some distance before leaving the eye so that it is hard to tell whether they decussate, but about half go to each tectum after they sort out in the base of the brain.

The cyclopean eyes of 340–2, 340–3, and 360–1 lack optic stalks and are even more closely related to the diencephalon than the preceding cases, in that the sensory





retina projects posteriorly well into the third ventricle, instead of simply being continuous with the diencephalic wall. The pigment epithelium, however, is again a reflection of the diencephalic wall. In 340–2 the optic fibers clearly decussate as they leave the retinal tissue, with a large bundle from the right side of the eye going to the left tectum and a bundle half as large from the left side of the eye going to the right tectum. Fish 340–3 also has a large fiber bundle going to the left tectum and a small bundle to the right tectum, but fiber paths as they leave the eye cannot be followed clearly enough to see whether they decussate. All of the optic fibers of 360–1 go to the left tectum.

Anophthalmic fish 109–1 has no eye tissue identifiable in section.

Discussion

The two synophthalmic fish that responded directionally to the optokinetic drum in the same manner as control fish were found to have optic nerves that distribute equally to the two optic tecta after decussating at least in part, and possibly entirely. The synophthalmic fish of similar external appearance that failed to respond to the drum was found to have optic fibers distributing solely to the telencephalon on one side, and was probably blind.

The failure of the perfect cyclopean fish to respond to the drum is harder to explain. Until the histological sections of the first season were studied, it was thought possible that in the four cases tested the optic nerve fibers from each side of the eye just happened to distribute about equally to both optic tecta. Horizontal optokinetic reflexes might then be cancelled out. This cannot be the explanation, however, because two of the total of eight perfect cyclopean fish tested have optic nerve fibers distributing to the optic tectum of one side only. No other explanation for the failure to respond is apparent. A right or left eye, even when displaced far toward the midline (fish 76–5), will send impulses that will initiate reflexes in response to the drum. Therefore, the two pupils or two separate retinal arcs of the typical synophthalmic condition are not requisite for the response. There is slight evidence that the cyclopean fish can see objects placed close in front of them. Comparison of their behavior with that of anophthalmics when objects are introduced near them, leads to the belief that the cyclopeans can see, but does not afford rigorous proof. If they can see, the failure to respond to the drum remains perplexing.

The individual with only one functional eye in the synophthalmic position and nerve distributing only homolaterally, responded directionally to a rotating drum in the same way as one-eyed anurans in which the regenerating optic nerve had been forced to grow to the homolateral optic centers (Sperry, 1945). Thus, Sperry's results in a regenerative situation are extended to a primary developmental situation.

All figures are photomicrographs of Bodian preparations.

Figure 6. Transverse section through synophthalmic eyes of fish 89–2. Note ventral fusion of sensory retinal arcs. × 115.

Figure 7. Transverse section through perfect cyclopean eye of fish 105–1. Nasal pits are above, tubular mouth below. × 115.

Figure 8. Transverse section of portion of eyes of fish 76–5. The large left eye sends a normal-sized nerve to the left side of the brain. × 800.

SUMMARY

1. Perfect cyclopean, closely synophthalmic, and anophthalmic fish hatchlings were obtained by magnesium chloride treatment or by hybridization.

2. Synophthalmic fish, with distribution of optic nerve fibers generally similar to controls, responded essentially normally to a horizontally rotating optokinetic drum.

3. A synophthalmic fish, with optic fibers distributing to the telencephalon of one side, failed to respond to the drum.

4. A fish with only one functional eye in the synophthalmic position, and optic fibers distributing entirely homolaterally, responded in the *opposite* direction when the drum was rotated toward the blind side.

5. Although there was other, somewhat inconclusive, evidence that they could see, eight perfect cyclopean fish failed to respond to a horizontally rotating drum.

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THE REVERSIBLE REPLACEMENT OF POTASSIUM BY RUBIDIUM IN ULVA LACTUCA [1]

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The substitution of rubidium ion for potassium ion in physiological processes has been studied for over 80 years. This subject is of special interest in view of the close chemical similarities of the two ions, rubidium being much more akin to potassium than is sodium. Permeability studies have revealed a high penetration rate of both rubidium and potassium ion (Brooks, 1932, 1939).

Studies on the replacement of potassium by rubidium have compared, on the one hand, the amount of physical replacement of potassium by rubidium within the cell, and on the other hand the suitability of rubidium as a substitute for potassium physiologically. Complete physical replacement of potassium by rubidium has been shown for *Chlorella* (Pirson, 1939), and yeast (Scott, unpublished data); partial physical replacement occurs in vertebrate muscle (Follis, 1943; Heppel and Schmidt, 1938; Mitchell, Wilson and Stanton, 1921) and in the duckweed *Lemna minor* (Pirson and Kellner, 1952). Rubidium can completely substitute for potassium in the repolarization of nerve (Feng and Liu, 1951; Gallego and De No, 1947), the restoration of heart beat in the frog heart (Ringer, 1884; Zwaardemaker, 1919), the activation of spermatozoa (White, 1953), the transport into the erythrocyte (Love and Burch, 1953), the activation of zymase (Giordani, 1932), the respiration of mitochondria (Pressman and Lardy, 1952), the bacterial production of pyruvic acid from malic acid (Lwoff and Ionesco, 1947), the activation of pyruvic phosphoferase (Kachmar and Boyer, 1953), and for growth in the bacterium *Streptococcus faecalis* (MacLeod and Snell, 1948). Rubidium is less effective than potassium in supporting assimilation, chlorophyll formation and cell division in *Chlorella* (Pirson, 1939), in supporting growth in *Lactobacillus casei* (MacLeod and Snell, 1948), or *Nitzschia closterium* (Stanberry, 1934), or for the secretion of adrenalin (Hermann, 1942). Rubidium is ineffective in supporting antibiotic activity in the subtilin-producing strain of *Bacillus subtilis*, although it promotes growth as well as does potassium in this bacterium (Feeney and Garibaldi, 1948). The element is definitely toxic at high concentration in certain bacteria, being antagonized by potassium (Scharrer and Schropp, 1933), and is fatal to rats when it is substituted for up to 50–66 per cent of the potassium in the tissues (Follis, 1943; Heppel and Schmidt, 1938; Mitchell, Wilson and Stanton, 1921; Zipser and Freedberg, 1952).

Previous work has been concerned with the extent of replacement of potassium

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by rubidium or the physiological results thereof, rather than with the kinetics of replacement. The present work is a kinetic study of the reversible exchange of rubidium for potassium and of potassium for rubidium.

Materials and Methods

The organism selected for this study was the green alga, *Ulva lactuca*. This marine organism, like most cells living in a high-sodium, low-potassium environment, normally accumulates potassium and partially excludes sodium. Consisting of large membranous fronds two cell layers in thickness, thus presenting a large surface area for interchange with the environment, this alga is particularly well suited for investigation of this nature.

Large fronds were collected from Perch Pond, near Falmouth, Mass., and conditioned for at least 24 hours in running sea water under incandescent illumination. Samples, cut from the same frond, were given a brief rinse in isotonic sucrose followed by a blot in absorbent toweling to remove most of the adherent sea water, then placed in large finger bowls with about 160 ml. per sample of an artificial sea water (Marine Biological Laboratory, Formulae and Methods IV, 1956), containing rubidium instead of potassium. The rubidium sea water was replaced with fresh rubidium sea water after 50 hours. Diffuse illumination was present during the experiment, which resulted in a slight drop in rubidium sea water pH. After 96 hours in rubidium sea water, the samples were placed in running sea water for 120 hours. Samples were removed in triplicate at various times, rinsed in isotonic sucrose for 30 seconds and blotted three times in absorbent tissue to remove extra-cellular sodium, potassium and rubidium ion. Wet and dry weights were taken and cell water calculated by difference. The samples were ground, extracted with 1 N HNO_3 for two hours at 110° C., and the extracts diluted to volume in 50-ml. volumetric flasks. The extracts were analyzed for sodium, potassium and rubidium ions by the Beckman flame spectrophotometer.

Results

The initial uptake of rubidium is both rapid and nearly complete within the first four hours, the time at which the first samples were taken. At 96 hours the rubidium ion concentration reaches a maximum of about 87 per cent of the control value of potassium; longer immersion in rubidium sea water does not result in an increase above this maximal concentration value. The potassium loss is initially rapid and then continues to decrease in a manner parallel to that of the control, reaching a minimum of about 13 per cent of the control (Fig. 1).

When the fronds are placed in running sea water, the kinetics of the replacement of rubidium by potassium are quite different from those involving the replacement of potassium by rubidium. After an initial sharp drop in rubidium for 10 hours and a sharp rise in potassium for 10 hours, the rates of loss of rubidium and rise of potassium decrease. After 120 hours in running sea water the potassium content of the alga has practically reached that of the control, whereas the rubidium content has been reduced to only 30 per cent of its maximal value. Longer immersions were not practical, as the alga begins to show signs of aging.

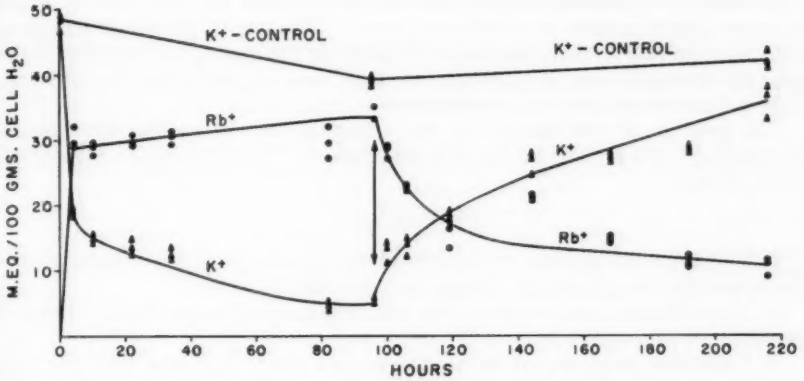

FIGURE 1. The replacement of potassium by rubidium within *Ulva lactuca* in rubidium sea water, and the replacement of rubidium by potassium on transfer of the alga to running sea water. The arrow indicates the time of transfer to running sea water. Concentrations are expressed on a cell water basis.

Throughout the experiment the sodium concentration of the samples remained essentially constant at 26 ± 4 meq. per 100 gm. cell water.

DISCUSSION

The initial uptake of rubidium is too rapid to be explained on the basis of a new, separate uptake mechanism for rubidium alone, for such a mechanism would have to be much faster than that previously demonstrated for potassium in this alga (Scott and Hayward, 1954b). Rather, the rate of rubidium uptake is entirely consistent with the known rate of potassium turnover at 20° (Scott and Hayward, 1954a). Therefore, rubidium is being transported by the same mechanism as is potassium. The cessation of rubidium uptake is probably the result of the establishment of an equilibrium between the rubidium and the potassium concentrations, for the total alkali metal base ($Na^+ + K^+ + Rb^+$) is constant within ± 5 meq. of that of the control (76–65 meq./100 gm. cell water).

Rubidium does not seem to affect the final level of potassium re-accumulation, but it would appear to have an effect on the kinetics of potassium re-accumulation. The re-accumulation of potassium is too slow to be accounted for solely on the basis of exchange of potassium ion for rubidium ion. Rather, it would seem that two factors might be operative: 1) the full maintenance of the potassium-accumulation mechanism is not supported by rubidium ion; 2) the age of the samples may be such as to render the postassium-accumulating mechanism sluggish. Since Ulva cycles from gametophyte to sporophyte generation on the order of every two weeks, it will be seen that the time course of the experiment (9 days) includes most of one phase of the cycle of the organism. (Whether a particular frond was gametophyte or sporophyte was not determined in this work, for the two generations are morphologically identical.)

In another experiment samples were left in rubidium sea water for as long as 144 hours before transfer to running sea water. The rate of potassium re-accumulation was identical to that found after 96 hours in the experiment reported above; hence the increased time in rubidium sea water was not progressively detrimental to the potassium-accumulating mechanism.

The presence of rubidium within this organism did not prevent the formation and discharge of a germinal ridge in some samples. These samples were not used in the analyses.

Summary

1. Rubidium ion replaces two-thirds of the potassium of *Ulva lactuca* within four hours after being placed in rubidium-containing sea water.

2. The rubidium concentration does not increase more than 5 meq. during the remainder of the experiment.

3. Potassium re-accumulation in running sea water is slower than the initial exchange of rubidium ion for potassium ion.

4. The disparity in the exchange kinetics is discussed.

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THE MORPHOLOGY AND LIFE-HISTORY OF THE DIGENETIC TREMATODE, MICROPHALLUS SIMILIS (JÄGERSKIÖLD, 1900) BAER, 1943 [1]

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Although significant observations had been reported earlier, the first complete life-histories of microphallid trematodes were worked out by Cable and Hunninen (1940) and Rankin (1940b). M'Intosh (1865) had described and figured larval worms from the tissues of the green crab, *Carcinides maenas* (Linnaeus), taken at St. Andrews, Scotland, but the life-cycles of trematodes were quite unknown at the time and M'Intosh described the structures as eggs, each of which contained a tiny worm that he surmised became a sexually mature distome in "such fishes as the Cotti, Gadi, and others," which feed on the crustaceans. He reported a specimen of *Cottus bulbalis*, about a foot long, with (p. 204) "two entire specimens of *Carcinus maenas*, each upwards of two inches across the carapace, in its stomach, besides the partially digested debris of others." His descriptions and figures identify the worms as members of the genus *Microphallus* and with considerable certainty as *M. similis* (Jägerskiöld, 1900). Although measurements were not given, his Figure 5 of an excysted specimen shows the suckers to be of approximately equal size and the "small globule" (seminal vesicle) anterior to the acetabulum, together with the size of the gonads, evidence full development of the metacercaria.

Levinsen (1881) described adult trematodes from the eider duck, *Somateria molissima*, taken at Egedesminde, Greenland, as *Distoma pygmaeum* and Brandes (1889) described similar worms from *Tringa alpinus* as *Distoma claviforme*. Jägerskiöld (1900) described specimens from Swedish gulls, *Larus argentatus* and *L. fuscus*, as *Distoma pygmaeum* var. *simile*. Other species have since been described from different hosts in other parts of the world. Ward (1894) described worms from American lake-fish as *Distoma opacum* and noted their similarity to *D. pygmaeum*. The parasites occurred in *Amia calva*, *Ictalurus punctatus* and *Perca flavescens*, and encysted larval stages were found in the crayfish, *Cambarus propinquus*. With the dismemberment of the old genus *Distoma*, Stossich (1899) erected the genus *Levinsenia* to include *Distoma brachysomum* Creplin, 1837, *D. macrophallos* von Linstow, 1875, *D. pygmaeum* Levinsen, 1881, and *D. opacum* Ward, 1894. Lühe (1899), but not Looss (1899), as has been claimed (cf. Looss, 1902: p. 704) designated *L. brachysoma*, and Jägerskiöld (1900) proposed *L. pygmaeum*, as type of the genus *Levinsenia*. Noting differences between *D. brachysomum* and *D. opacum*, Ward (1901) named the latter species type of a

[1] The experimental work was done at the Marine Biological Laboratory, Woods Hole, Massachusetts, during the period May 1 to October 31, 1956.

new genus, *Microphallus*. In this paper he reported that the name *Levinsenia* Stossich is a homonym of *Levinsenia* Mesnil, 1897, a polychaete annelid, and that Stiles and Hassall were to propose the name *Levinseniella* to replace it. Although the paper by Stiles and Hassall did not appear until 1902, the name *Levinseniella* Stiles and Hassall in Ward, 1901 was validated with *L. brachysoma* as type. Jägerskiöld (1901), although aware of the announcement by Ward, proposed the name *Spelotrema* for the invalid name *Levinsenia*, with *S. pygmaeum* as type. Cable and Hunninen (1940), commenting on this action, wrote (p. 153), "If the law of priority should be applied to this case, *Spelotrema* Jägerskiöld, 1901 should be suppressed as a synonym of *Levinseniella* Stiles and Hassall in Ward, 1901, since Jägerskiöld stated subsequently (1904) '*Spelotrema* (= *Levinseniella*)' and therefore certainly regarded them as synonymous. His later (1907) conception of two distinct genera is valid, however, and must be accepted although he should not have retained for them names which he had regarded previously as synonyms. To suppress *Spelotrema* as a synonym of *Levinseniella*, and propose a new generic name for the species at present allocated to the genus *Spelotrema*, would probably increase rather than diminish the present confusion. For this reason, the writers are inclined to let the matter stand." As noted, *L. brachysoma* and *S. pygmaeum* are not congeneric and the characteristic features of the two genera were presented clearly by Rankin in two papers (1939, 1940a). The problem of nomenclature, stated by Cable and Hunninen, was resolved when Baer (1943) suppressed *Spelotrema* as a synonym of *Microphallus* and transferred all species from the former genus to the latter one. Baer described *Microphallus gracilis* from *Neomys fodiens* and formulated a key to thirteen members of the genus. Strandine (1943) and Rausch (1946a, 1946b, 1947) discussed morphological features and host-specificity of species in the genus *Microphallus*. Rausch and Locker (1951) described *Microphallus enhydrae* n. sp., from the sea-otter, *Enhydra lutris*, and recognized fourteen species in the genus. Stunkard (1951, 1953) described metacercariae from the horseshoe crab, *Limulus polyphemus*, and their development to sexual maturity in white mice, golden hamsters, and the herring gull, *Larus argentatus*. Although the worms agreed closely with the description of *M. claviformis* (Brandes, 1899), bionomic features seemed to preclude their allocation to that species and they were described as members of a new species, *Microphallus limuli*. Cable and Kuns (1951) erected a new genus, *Carneophallus*, and discussed the evolution and interrelationships of genera in the family Microphallidae.

Lebour (1905) reported grape-like masses of "sporocysts" in the liver of *Littorina rudis* (= *L. saxatilis*), filled with tail-less cercariae, doubled up in a curious manner. When extended, they measured 0.25 mm. in length and the figure shows them to be microphallid metacercariae. Miss Lebour regarded these larvae as identical with the encysted worms found by M'Intosh (1865) in the green crab, *C. maenas*. Brandes (1889) had suggested that the larva in the green crab is the encysted stage of *Distoma claviforme*, described by him from *Pelidna* (*Tringa*) *alpina* and *Aegialitis hiaticula*. Nicoll (1906) described specimens from the ceca and intestine of *Larus argentatus* taken at St. Andrews, Scotland, which he identified as *Levinsenia similis*, the species that Jägerskiöld (1900) had described as a variety of *Dist. pygmaeum* and (1907) raised to specific rank as type of *Spelotrema*. Nicoll noted that his specimens were somewhat larger than those of

Jägerskiöld and that worms from the ceca were larger than those from the intestine. He declared that the metacercariae from *C. maenas* are as likely to prove larvae of *S. similis* as of *S. claviforme*. The following year, Nicoll (1907) described the worms from *L. argentatus* at St. Andrews as a new species, *Spelotrema excellens*, distinct from *S. similis*. Other specimens from *Pelidna alpina, Totanus calidris* and *Aegialitis hiaticula* were described as a new species, *Spelotrema feriatum*. He gave a redescription of *S. claviforme* and in a footnote stated that the larvae from *C. maenas* are larger than the adults of *S. claviforme* and must belong to another species, perhaps *S. excellens*. The smaller metacercariae described by Lebour (1905) from the liver of *Littorina rudis* were suggested as the encysted stage of *S. claviforme*. Lebour (1908) designated the metacercaria from *C. maenas* as *Cercaria carcini* and recognized the difficulty of relating the larval forms of *Spelotrema* with any known adults. Nicoll and Small (1909) examined crabs at Millport on the Scottish west coast during August, 1908. They found three of four *C. maenas* and one of five *Cancer pagurus* infected with encysted metacercariae which they identified as a species of *Spelotrema*, most probably *S. excellens* Nicoll. At St. Andrews every green crab was infected, often with every tissue and organ riddled with cysts which occurred sometimes singly and sometimes in clusters in the liver, gonads, and along the blood vessels, nerves and intestine. They noted, as had Lebour (1908), that the cysts varied in size, shape and thickness of wall. They measured cysts of three size groups; the small ones were oval with thin walls and the largest ones were spherical with thick walls composed of inner concentric and outer radial layers. Referring to the gradual increase in size of the cysts, the authors stated (p. 239), "From these figures there seems no reason to suppose that these groups are other than stages in the growth of the same cyst, and such being the case it is evident that the cercariae increase considerably in size during their sojourn in the crab." They measured a cyst from the original material of Prof. M'Intosh which was 0.29 mm. in diameter. They suggested a possible error in the measurement given in his (1865) report, mentioned also by Guyénot *et al.* (1925).

Lebour (1912) proposed that larval trematodes should be classified on bionomic grounds and arranged the British marine cercariae in two categories, dependent on whether they were produced in sporocysts or in rediae. Among those which develop in sporocysts, she gave a more complete account of *Cercaria ubiquita*, a larva which she previously (1907) had described from *Paludestrina stagnalis* at Fenham Flats and Loch Ryan on the west coast of Scotland. She reported that this species occurred also in *Littorina saxatilis* (syn. *L. rudis*), and at Millport the usual host was *Littorina obtusata*. The cercariae resembled *C. cellulosa* and *C. pusilla* of Looss (1896) and Lebour reported that they entered *C. maenas* and *Cancer pagurus* where they developed into "Spelotrema-like cercariae." The encysted larvae occupied (p. 432) "almost every tissue of the crab, liver, muscles, gonad and outside the blood vessels. Having settled down it grows considerably and the cyst with it, but the latter however is still very thin-walled. The stylet is lost when the cyst measures about 0.30 mm. across. The ventral sucker and alimentary canal appear and the body spines begin to form. The worm stops growing when the cyst is about 0.35 mm. across and then the cyst wall becomes very thick, 0.02 mm. thick, and the real resting stage begins. The cercaria is now of the ordinary *Spelotrema* form. The usual size of the thick-walled cyst is

0.4–0.48 mm. across." After describing the excysted metacercaria, Lebour stated (p. 433), "From what has been said there can be little doubt that *Cercaria ubiquita* is the young form of *S. excellens* the first host thus being *Paludestrina stagnalis, Littorina obtusata* and *L. rudis*. The intermediate host *Carcinus maenas* and *Cancer pagurus* and the final host probably the herring gull, *Larus argentatus*." In this paper Lebour also gave figures of *Cercaria carcini* Lebour, 1908 and *Cercaria minor* sp. inq., both from *C. maenas*. These species were encysted and therefore metacercariae; furthermore, since the cyst walls were thin and the worms somewhat smaller, it is probable, as suggested by Nicoll and Small (1909), that these larvae are identical with the ones identified by Lebour as *Spelotrema excellens*.

Guyénot, Naville and Ponse (1925) described metacercariae which they found in a single, formalin-preserved specimen of *C. maenas* taken at Boulogne-sur-Mer, France. The cysts were oval, 0.40 by 0.35 mm., and the larvae were identified as *Spelotrema carcini* Lebour. These authors accepted Lebour's (1912) account, designating *C. ubiquita* as the larva of *S. excellens*, but noted that when computed from the stated magnification of the figure, the metacercaria portrayed by M'Intosh was only 0.13 by 0.16 mm. As noted earlier, M'Intosh gave no measurements and the presumed error in magnification may be explained by reduction in size of the drawing on publication. The French authors also described larger cysts, 0.90–1.2 mm. in diameter, in which the walls were weakened and the trematode larvae were filled with spores of a microsporidian, *Nosema* (*Plistophora*) *spelotremae* n. sp.

Stunkard (1932) described cercariae from *Littorina saxatilis* and *L. littorea* at Roscoff, France as *Cercaria ubiquitoides*. Although the larvae were very similar to *C. ubiquita*, slight differences between these specimens and Miss Lebour's description prevented their allocation to that species. In *C. ubiquita* Lebour described two ducts on each side, "which run up the body springing from two masses of large cells which occupy the greater part of the body." In her figure, Lebour showed six gland cells on each side with the two median ducts crossing to open on opposite sides of the body. The cercariae at Roscoff were described as having four penetration glands on each side of the body, with ducts which lead forward, three associated in a common bundle that passes along the lateral face of the oral sucker while the other duct lies more mediad and passes over the sucker. The penetration glands did not stain with neutral red but the secretory granules were clearly visible. These granules, colorless in the cell bodies, stained a deep blood-red in the terminal portions of the ducts and frequently accumulated there to form enlargements. Stunkard also described metacercariae from *C. maenas* and *Porcellana longicornis*, which were referred provisionally to the genus *Spelotrema*, but no attempt was made to relate them to *C. ubiquitoides*.

Rees (1936) described an ubiquitous cercaria from *L. rudis, L. obtusata* and *L. littorea* collected at Aberystwyth in February and March, 1935. He noted that the larvae were almost identical with those described by Stunkard (1932) from *L. rudis* and *L. littorea* at Roscoff. There were slight differences in measurement and other differences concerned the penetration glands, which in the specimens studied by Rees were, "more distinct, not lobed and the most anterior pair of cells is separated from the other three pairs." Rees declared (p. 621), "It is difficult to determine whether these differences are individual or specific because there are so few larval characters in cercariae of the Ubiquita group which can be

used for separating species. The problem is rendered more difficult by the close resemblance of the adult trematodes (species of the genus *Spelotrema*)." In general, the measurements given by Rees agree with or overlap those given for *C. ubiquitoides,* except for the length of the stylet which measured 28 microns against "about 25 microns" in *C. ubiquitoides.* The size, shape and relative position of the gland cells are altered by degree of maturity of the larva, by contractions of the body musculature, and by the emission of secretion into their ducts. Since the ducts from the most anterior pair of penetration glands are separate from the ducts of the other cells, the cell bodies may be somewhat removed. The observation by Rees that only the two anterior pairs of cells and ducts take up neutral red stain, whereas the others do not, is a significant contribution to knowledge of the species. Obviously, I failed to note this feature in *C. ubiquitoides,* but the staining reaction differs with the constitution, concentration, age and condition of the neutral red solution. Accordingly, I am disposed to regard the larvae described by Rees and *C. ubiquitoides* as specifically identical. Since each penetration gland has its own duct, the account of Lebour is not precise and the lateral one of the two reported ducts on each side of the body is almost certainly a bundle of three ducts. Furthermore, it appears that *C. ubiquitoides* can not be distinguished from *C. ubiquita* Lebour, 1907, and the name should be suppressed as a synonym. Lewis (1926) had reported *Spelotrema simile* as very common in gulls of the Aberystwyth area and the finding was confirmed by Rees (1936) who stated (p. 624), "The unusually high percentage infestation of *Littorina rudis* with this cercaria, together with the high percentage of gulls parasitized by *Spelotrema simile,* suggests that this may be the larval form of this species."

Cable and Hunninen (1938) described the successive stages in the life-cycle of a trematode which they identified as a new species, *Spelotrema nicolli.* The asexual generations were in the snail, *Bittium alternatum,* the metacercariae in the blue crab, *Callinectes sapidus,* and the sexual generation was developed in young herring gulls, *Larus argentatus.* These authors (1940) gave a more complete description of *S. nicolli,* the adult of which was compared with that of *S. pygmaeum, S. claviforme, S. simile, S. excellens* and *S. brevicaeca.* In size and size of organs, *S. nicolli* agrees closely with *S. simile;* the major difference is in the size of the male papilla, which is much smaller and similar to that of *S. pygmaeum.* The metacercariae were found only in certain slender fibers which extend from the viscera to the bases of the legs. Cysts increase from 0.05 to 0.50 mm. in diameter; the metacercariae become almost as large as the adults; the excretory formula is 2 [(2 + 2) + (2 + 2)], which persists in the adult stage. The cercaria agrees closely with the description of *C. ubiquitoides* and of the species described by Rees; the major difference is the shorter length of the stylet. *Spelotrema nicolli* is identified by the size of the male papilla, the location of the metacercariae, and the taxonomic position of the first and second intermediate hosts.

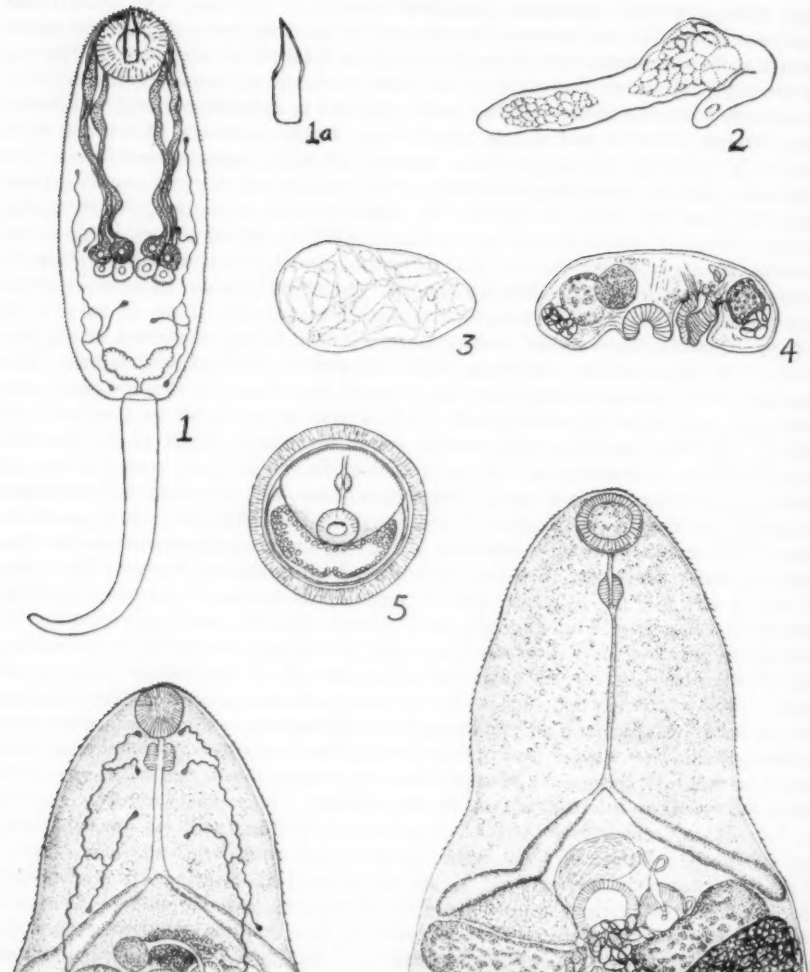
Timon-David (1949) reported metacercariae from the hepatopancreas of *C. maenas* in the Mediterranean at Marseille. The cysts were oval and on this feature and their size, they were identified as *Spelotrema carcini* Lebour.

The present report covers part of a project on clam investigation conducted by the U. S. Fish and Wildlife Service. Since the green or shore-crab, *C. maenas,* is a serious predator of *Mya arenaria* on the New England coast, a survey of its parasites was undertaken in an attempt to determine whether or not some of them

might serve as possible means of biological control. It has long been known that *C. maenas* in the Woods Hole area is infected by an undetermined, encysted metacercaria and experiments have been conducted to discover its identity, life-history, and biology. Metacercariae were fed to white mice and excysted specimens (Fig. 6) recovered after 24 hours. Other cysts were fed to recently hatched, uninfected birds, *Sterna hirundo* and *Larus argentatus*. Large numbers of worms were recovered, including all stages from juvenile to fully mature specimens. The structure of the metacercariae, especially of the smaller and recently excysted ones, suggested that they may be specifically identical with a minute, stylet-bearing cercaria (Figs. 1, 1a) reported by Stunkard (1950), which occurs in *Littorina obtusata, L. saxatilis,* and rarely in *L. littorea.* Small green crabs were exposed to these cercariae and became heavily infected; enormous numbers of worms entered their tissues and developed to metacercariae, identical with those of natural infections. Small crabs exposed continuously with six to eight infected snails died in 10 to 20 days, and on dissection, each one yielded thousands of larvae. The parasites were present throughout the body of the crab, but the heaviest concentration was in the digestive gland. When first encysted, the cyst is oval, the wall is very thin, and the stylet persists for a long time. Eggs of the parasite, recovered from the droppings of terns and gulls, and others teased from the uteri of gravid worms, were used to infect specimens of *L. obtusata,* collected near the laboratory from an area which is not frequented by birds and where examination of 200 snails showed no infection. The eggs were embryonated under running sea water for seven days and then spread on fronds of *Fucus* which were allowed to partially dry to ensure that the eggs would become attached to the slimy surface of the alga. These fronds were placed with 10 small specimens of *L. obtusata* in a gallon jar with a small opening and sharply curved upper shoulders, half filled with sea water and provided with a stream of fine bubbles from an air pump. The water was not changed for ten days and subsequently on alternate days the top water was siphoned off and replaced with fresh sea water. Since the eggs of microphallid trematodes do not hatch until they are eaten by a suitable snail, and since from the design of the experiment it is impossible to tell when eggs were eaten, the age of an infection is not known precisely. The snails were first exposed on July 31, and on October 1, five snails that were alive in the jar were crushed and examined. Three of them were infected; two with large numbers of small daughter sporocysts but not yet producing cercariae, and the other contained three primary or mother sporocysts (Fig. 2) but no free daughters. The early stages of the infections afford clear evidence of their experimental nature and the complete life-cycle was thus consummated by laboratory infection of both intermediate and final hosts.

The adult worms agree so completely with the descriptions of *Microphallus similis* (syn. *Spelotrema simile*) as given by Jägerskiöld (1900) and Odhner (1905), that they are assigned to that species. The experimental demonstration of the life-cycle confirms the suggestion of Rees (1936), that *Cercaria ubiquita* Lebour is the cercarial stage of *M. similis.* Actually, the adults were described by Jägerskiöld (1900), the metacercariae by M'Intosh (1865), and the cercariae by Lebour (1907). Whether the specimens described by Nicoll (1907) as *S. excellens* and *S. feriatum* are specifically distinct remains to be determined.

Attempts to infect *Limulus polyphemus* were unsuccessful. Three small horse-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shoe crabs with carapace widths of 17, 19, and 21 mm., collected near Orleans, Massachusetts, were exposed to cercariae for two weeks. On dissection, there were no recently entered, unencysted larvae although all three harbored mature cysts of *Microphallus limuli* in their livers. This finding supplements that of Stunkard (1953), who reported full-sized cysts in small *L. polyphemus*. In paragraph 3, line 4, of that report, 3 mm. should read 30 mm.

Description of Stages in the Life-Cycle

Measurements in millimeters

Adults (Fig. 7)

Egg-bearing specimens from gulls and terns are about the same size. Fixed, stained and mounted they measured: length, 0.36–0.7; width, 0.22–0.36; acetabulum, 0.048–0.065; oral sucker, 0.05–0.065; pharynx, 0.02–0.03; male papilla, diameter 0.038–0.058; testes, 0.10 × 0.06 to 0.16 × 0.09; seminal vesicle, 0.06 × 0.04 to 0.09 × 0.064; ovary, 0.08 × 0.05 to 0.1 × 0.062; eggs colorless on the ovarian side, yellow on the antovarian side, 0.022–0.027 × 0.011–0.012, often collapsed and somewhat distorted in fixed and stained specimens.

The body is oval to pyriform and either end may be wider; often there is a slight constriction between the more mobile forebody and the inert hindbody which is filled with reproductive organs and eggs. The dermomuscular wall is thin and weakly developed; it consists of the usual circular, longitudinal and oblique layers, but the fibers are delicate and relatively few. The edges of the forebody tend to turn ventrad, forming an adhesive cup. The cuticula bears flattened spines which are conspicuous on the anterior half of the body but become smaller and sparser posteriorly. The suckers are approximately equal in size; either may appear larger, depending on the degree of contraction by the sphincter and the size of the orifice. The esophagus is long and when the body is extended, it may be much longer than the ceca. The ceca are almost straight; they diverge at an obtuse angle and terminate at the acetabular level. The anterior tips are constricted and lined with cuticula, continuous with that of the esophagus. The excretory system is unchanged from the metacercarial stage and has the formula $2[(2+2)+(2+2)]$. The testes are dorsal, lateral, opposite; the vasa deferentia arise at the medial, anterior faces, pass mediad and anteriad where they unite to form the seminal

Figure 1. Cercaria from *L. obtusata*, free-hand drawing of specimen stained with Nile blue sulphate; 1a, lateral aspect of stylet.

Figure 2. Primary sporocyst from *L. obtusata*, experimental infection; fixed and stained specimen, length 0.30 mm.

Figure 3. Daughter or secondary sporocyst from *L. obtusata;* natural infection; fixed and stained specimen, length, 0.375 mm.

Figure 4. Cross-section of a mature worm to show the relative position and size of the acetabulum and of the male papilla. The metraterm opens into the genital atrium near the base of the papilla and in front of the left testis whose anterior portion appears in the section; width of the worm, 0.28 mm.

Figure 5. Metacercaria from *C. maenas*, natural infection; cyst 0.46 mm. in diameter.

Figure 6. Specimen, much flattened to study the excretory system, from intestine of a white mouse, 24 hours after the cyst was eaten; length 0.55 mm.

Figure 7. Adult specimen from intestine of *Sterna hirundo*, experimental infection; one of the largest specimens, flattened under a cover glass, fixed and stained, length 0.69 mm.

vesicle, an oval sac which lies anterior and dorsal to the acetabulum. A coiled ejaculatory duct, enclosed in glandular cells, leads from the vesicle to the muscular male papilla which almost fills the genital atrium, situated at the left of the acetabulum. The ovary is oval to triangular, located on the right side of the body, between the seminal vesicle and the testis and cecum of the right side. The oviduct arises from the median posterior region, coils posteriad and ventrad where it expands to form a fertilization space from which Laurer's canal winds to the dorsal surface of the body, opening in the midline just behind the acetabulum. The oviduct then turns dorsad and anteriad, receives a short common vitelline duct and enlarges to form the ootype, lined with cilia and enclosed in the cells of Mehlis' gland. The vitellaria are large, lobed glands, situated below and behind the testes; ducts from the two sides pass mediad, anteriad and dorsad, uniting to form the common vitelline duct which discharges into the initial portion of the ootype. The uterus passes backward from the ootype almost to the posterior end of the body and then loops forward in coils on the ovarian side of the body as far as the end of the digestive cecum, then backward almost to the posterior end of the body where it crosses to the opposite side and forms a corresponding series of loops on the left side, with the terminal metratermal portion emptying into the left side of the genital atrium (Fig. 4). The extent of the uterus anteriorly is determined by the number of eggs; in certain specimens the uterine coils may be below and behind the testes whereas in others the coils may underlie the ends of the digestive ceca.

The egg and miracidium

Egg-production begins almost immediately after the metacercaria is eaten by the final host. Figure 6 shows a worm which, fed as a metacercaria to a white mouse twenty-four hours earlier, already has eggs in the initial portion of the uterus. At first the egg-shell is thin, flexible and transparent; but as the eggs traverse the uterus, the shells become thicker, harder, and bright yellow. This coloration of the shell obscures the larva in living eggs, but development can be followed by study of serial sections of gravid worms. The egg is operculate and the ovum is situated toward the opercular end of the egg. In eggs near the metraterm, the miracidium appears to be fully formed, but eggs used for infection experiments were kept for a week in running sea water to insure fully mature larvae. The miracidia emerge only after the eggs have been ingested by the snail host.

Sporocyst generations (Figs. 2, 3)

The amount of experimental material is limited, but three primary sporocysts were removed from a specimen of *L. obtusata* two months after exposure to eggs of *M. similis*. These sporocysts were sluggish, oval to cylindrical and 0.25 to 0.40 mm. long. One of them, fixed and stained, is shown in Figure 2. The presence in them of recognizable daughters identified them as sporocysts of the mother or primary generation. Daughter sporocysts obtained from two experimental infections were young, small, and very numerous. Daughter sporocysts of natural infection measured 0.10 to 0.60 mm. in length; they are oval, occur in

large numbers, more than one hundred in a single snail. The wall often contains a yellowish pigment; it is thin and in older sporocysts change of shape results from movement of contained cercariae. Figure 3 was made from a daughter sporocyst, 0.375 mm. long.

Cercaria (Figs. 1, 1a)

Body length, 0.1–0.22 mm.; width, 0.02–0.05 mm.; the larva is very thin, delicate, colorless. The tail is 0.01–0.012 mm. wide at the base; contracted it is 0.05 mm. long, with fine cuticular annulations; extended it may be 0.25 mm. long and very slender. There is no acetabulum; the larvae move by strokes of the tail but are unable to creep. They swim upward and sink when motionless. In swimming the body is contracted, bent ventrad, while the tail is extended and lashes violently. The body is covered with cuticular spines and the dorsal wall of the sucker bears a stylet, 0.023–0.026 mm. long and 0.009 mm. wide. The stylet (Fig. 1a) is asymmetrical in lateral aspect. The oral sucker measures 0.025–0.032 mm.; other parts of the digestive system are not yet developed. The body contains numerous cystogenous glands and on either side, near the middle, there are four penetration glands. The two anterior cells on each side differ from the posterior ones; the difference is demonstrated by the use of neutral red or Nile blue sulphate solutions, especially the latter, which stain the secretory granules of the anterior cells selectively while the contents of the posterior cells do not take the stain. Ducts from the anterior pair of cells pass forward beside those from the other cells for a short distance, but about halfway to the mouth they separate from the others and pass more mediad, crossing the dorsal side of the oral sucker, while the ducts from the other three penetration gland cells form a bundle that continues forward and passes at the side of the sucker. The ducts from the two anterior pairs of cells open to the surface ventrally, below the tip of the stylet, whereas the ducts from the posterior pairs open more anteriorly and at the sides of the tip of the stylet. The excretory system is shown in Figure 1; the vesicle is U- or V-shaped and the flame-cell formula is $2[(1+1)+(1+1)]$. The cercariae are carried by respiratory currents into the gill chambers of *C. maenas* where they enter the body at the bases of the gills and possibly at other non-cuticularized places, pass by way of the vascular system to all parts of the body, and localize principally in the connective tissue of the digestive gland.

Metacercaria (Fig. 5)

The cysts increase in size and measure from 0.05 to 0.55 mm. in diameter; when the cercariae encyst they are bent ventrally; the cyst is oval and the wall is very thin and flexible. The stylet is retained for some time and readily identifies the species. As the metacercaria grows, the digestive tract and actabulum are formed and the number of flame-cells is doubled. As the worm grows, the cyst becomes spherical and the wall increases in thickness. In a full grown cyst, the cavity may be 0.35–0.40 mm. in diameter and the wall consists of two layers, an inner hyaline one which may be resolved into strata and attains a thickness of 0.02 mm., and an outer radially striated layer which increases to a thickness of 0.06 mm. The substance of this layer, after digestion in a pancreatin solution,

appears to consist of parallel prisms. The worms become almost full grown as metacercariae; the adults increase in size only by the further activity of the reproductive organs and the accumulation of eggs in the uterus. When younger and smaller metacercariae are eaten, the worms become gravid at a smaller size than when the metacercariae are older. The excretory vesicle of older metacercariae contains spherical concretions, the excretory wastes accumulated during the period of encystment.

Summary

1. The life-history of *Microphallus similis* has been worked out by experimental infection of both intermediate and final hosts.

2. Encysted metacercariae from *Carcinides maenas* developed to sexual maturity in *Larus argentatus* and *Sterna hirundo*. Eggs of the parasite developed in these hosts were used to infect *Littorina obtusata*. Two generations of sporocysts were recovered.

3. *Littorina saxatilis* and *Littorina littorea* also harbor the asexual generations at Woods Hole, Massachusetts.

4. The cercariae are minute, stylet-bearing monostomes and small green crabs, *C. maenas*, exposed to these cercariae became heavily infected; enormous numbers of larvae entered the tissues and developed into metacercariae identical with those of natural infections. Small crabs, each exposed continuously to the cercariae from six to eight infected snails, died in ten to twenty days and on dissection each yielded thousands of larvae.

5. The stages in the life-cycle of the parasite agree with descriptions by European investigators of corresponding stages: the metacercariae with metacercariae from *C. maenas*, described but not named by M'Intosh (1865); the adults with *M. similis* from Swedish gulls, described and named by Jägerskiöld (1900); and the cercariae with *Cercaria ubiquita* Lebour, 1907. The identity of these parasites as stages in the life-cycle of a single species is predicated.

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